

**Chick embryo heart cells in culture:
Behaviour in various sera, inherent growth
capacities, and differentiation potentialities.**

by

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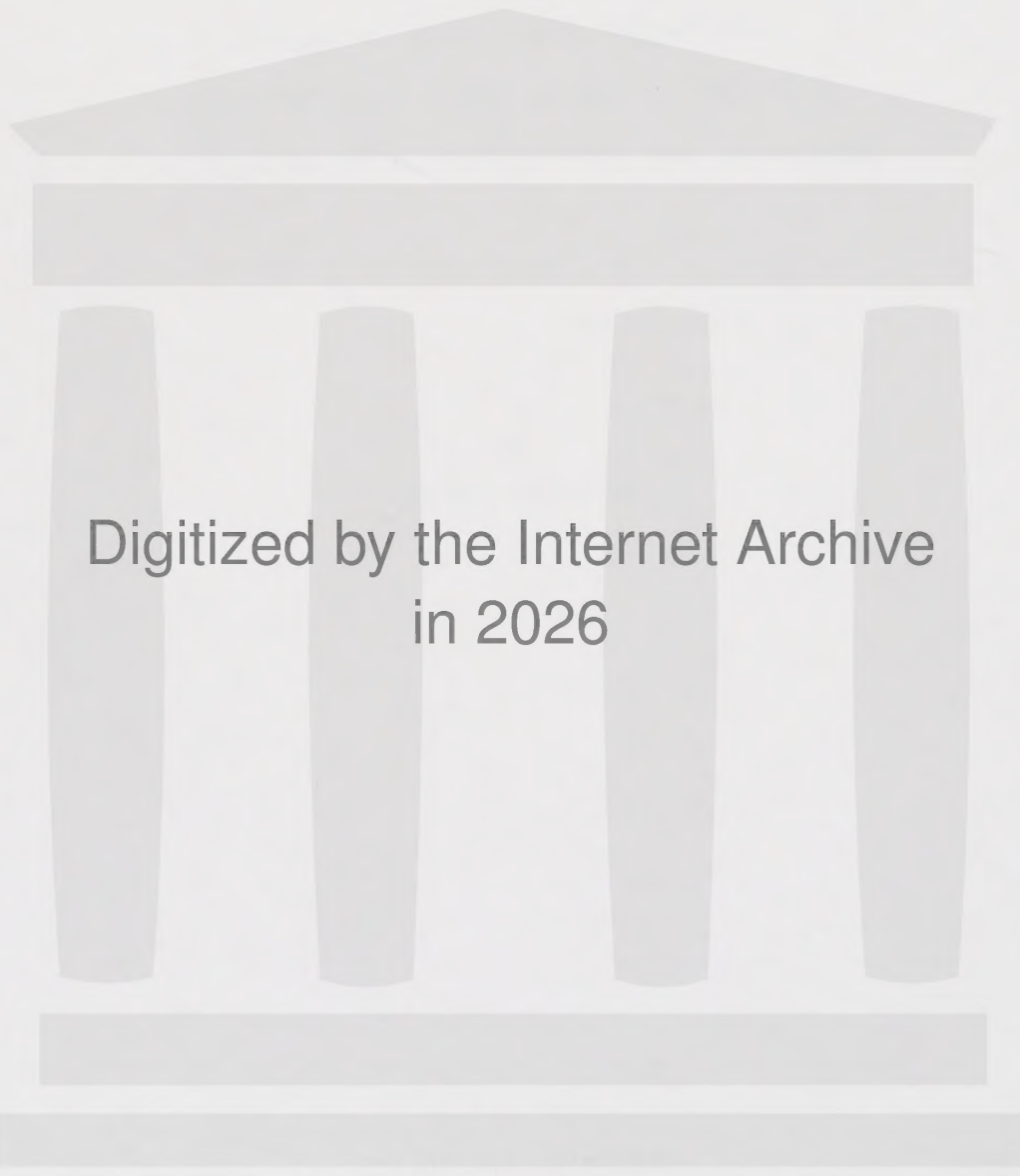
Lund 1975

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CAPACITIES, AND DIFFERENTIATION POTENTIALITIES.

av
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ABBREVIATIONS

Alb	= albumin
A/G	= albumin/globulin ratio
α, β, γ	= alpha, beta and gamma globulins
BM	= basal medium (30% serum + 70% Tyrode's BSS)
BSS	= balanced salt solution
c.d.	= cell density
D	= denatured (by heat)
DH.S.	= denatured horse serum
d.c.	= differentiated cell(s)
% d.c.	= % of differentiated cells/dish
dial	= dialysed
EE	= embryo extract
DEE	= denatured embryo extract
NEE	= normal embryo extract
F-cells	= fibroblast-like cells
GM	= growth medium (20% serum + 10% EE + 70% Tyrode's BSS)
g.r	= the exponential growth-rate
G.Symp	= general symptoms
HMW	= high molecular weight
H.S.	= horse serum
LMW	= low molecular weight
M-cells	= muscle cells
N	= normal (not dialysed or denatured)
n.b.	= newborn
S	= serum
% stretch	= % of stretched cells/dish
T.P.	= total protein
5-, 12-, and 18-day cells	= heart cells isolated from 5-, 12-, and 18-day chick embryos

GENERAL INTRODUCTION

The processes of cellular division, growth, and differentiation that take place during the embryological developmental period are basic phenomena in developmental biology. In vitro cultivation of embryonic myocardial cells is a well-known and useful method for studying these basic phenomena. Moreover, it provides an ideal cell type for investigating problems concerning inherent growth capacities, de-differentiation versus re-differentiation, antagonism between cellular proliferation and differentiation, contact inhibition of cellular movement and cell division, mechanism of spontaneous contraction and structural differentiation, growth control systems, the physiological responses of cells to variations in the environmental conditions, and other functional and metabolic activities.

The extensive literature on this subject, published during more than 60 years, makes possible intensive study and critical reference. Since Burrows in 1910 (13) introduced the in vitro cultivation of minced fragments of embryonic chick hearts in plasma clot, many authors have, with this technique, collected valuable data on specific functions and general activities etc. that take place in heart cells in culture. Thus, utilizing the above explant culture technique, Carrel (15) compared the inherent growth energy of chicken heart tissues from 10- and 17-day embryos and found a marked difference in the inherent activities of the two tissues, referable to the difference in the age of embryos that they were derived from. Parker (74), studying the functional differences determined by the origin of the chick-heart explants and the age of the donor, found that the rate of multiplication is not gradual in respect of age of the donor.

Abercrombie et al. (1, 2), observing social behaviour of heart cells in explant cultures, noticed the inhibition of cell movement in close-neighbour cells, "contact inhibition of movement", which consequently affected the growth of the cells in dense cultures (52, 12).

In 1952, Moscona (69) introduced the tryptic digestion of tissues into their separate single cell component. Cavanaugh (16), after applying this method for the preparation of single cells from embryonic heart tissue, stated "this procedure offers an opportunity to study the cells as free individuals as well as members of a population." In order to develop a method

of optimum conditions for the isolation and quantitative cultivation of embryonic cells, chick embryonic heart cells were subjected to an extensive study by Rinaldini (84) who, among other things, concluded that the growth in this system antagonizes functional differentiation.

Rumery et al. (88, 89), who studied growth, behaviour, and differentiation of myocardial cells from early chick embryos, concluded that the formation of striation in myofibrils preceded contraction. Agrell (4) demonstrated an antagonism between mitotic activity and visible differentiation by using cell-cultures from early embryonic chick hearts. Furthermore, in the same study, he concluded that myogenesis appears as a reversible process which could be induced or suppressed, depending on the qualities of the medium. The percentage of spontaneous contracting heart cells under variable conditions in culture was extensively studied by DeHaan (20) who found, among other things, that this percentage depends on a wide variety of environmental parameters; moreover, he distinguished two morphological cell types in heart-cell cultures from various embryonic ages.

By cloning single heart muscle cells from 5-day chick embryos, Chacko (17) showed that, contracting cells having cross-striated, myofibrils undergo mitosis in vitro and give rise to spontaneously beating daughter cells.

The total growth capacity of various kinds of cell cultures has been very little studied. Interest has been focused on the varied growth rate during the exponential growth phase. Through work with amoeba cells (3), Agrell showed that the growth capacity of these cells could depend on the inoculum size, and he and the present author then decided to test this dependent growth capacity on disintegrated chicken heart myoblasts (Chapter 2).

Changes are known to occur in embryonic cells correlated with differentiation and maturation (51). Earlier authors, using "explant-culture", have evaluated growth capacities of cells from donors of different developmental ages. Variable results were obtained. Thus Carrel (15) and Medawar (63) found that growth capacity in explants of heart tissues declines with embryonic developmental age, whereas Hoffman et al. (42) observed similar growth capacities in explants of embryonic and one-year old chicken hearts. Similar mitotic indices were obtained by Chaytor (18), who compared explants of chicken heart tissue from 7- and 14-day embryos. On the other hand, Parker (74) showed that growth rate of various tissue-explants does not correlate with gradual increase in embryonic age.

Therefore, the present investigation evaluated the growth capacities of embryonic cells related to their respective developmental age in a "disintegrated tissue" system (Chapter 2). During the course of this study, some positive results were obtained which pointed to such a possible correlation. Consequently, a new question arose: Is this correlation really a result of different inherent growth capacities of myocardial cells, or is it an artifact caused by the selection of cell types in culture consequently having different growth capacities? Myocardial cells in culture can be characterized by manifesting two main properties, i.e. functional differentiation and structural differentiation. On the other hand, factors such as proliferation, quality of the medium, and cell density are known to affect the exhibition of these properties in culture. Therefore, attempts were made to follow the effect of each factor on cell differentiation in this system (Chapter 3). Moreover, optimum conditions in which the heart cells could manifest maximum differentiation were sought in the course of this study (Chapter 3).

From the earliest beginning of tissue-culture technique up to present, natural media, such as plasma clots, sera, and tissue extracts, have been used solely or supplementary for the maintenance and growth of animal cells in culture. Among the natural media, serum is the traditional biological fluid and the one most commonly used (75). Therefore the effects of serum on cell-cultivation have been the object of a large number of studies, the results being variable. To study the behaviour of chicken heart cells in culture, from the aspect of their embryological age (Chapter 2 and 3), it was necessary to perform the experiments under controlled conditions. As serum constitutes the main part of the nutritional medium, and by its nature is a very complex (7) biological substance, it inevitably exerts various actions on cells in culture. It was therefore desirable to approach a better understanding of its nature and its influence. To establish a criterion for possible correlation between serum character and its growth promoting or toxic factors, a wide range of sera, representing different origins and donor ages, and different treatments, was tested (Chapter 1). Moreover, to find out to what extent the effects of serum on one cell type are applicable for other cell systems, two additional established cell lines (Chinese-hamster cells and Ehrlich cells, see Material and methods, Chapter 1) were included in this study (Chapter 1).

CHAPTER 1

EFFECT OF VARIOUS SERA ON BEHAVIOUR OF CELLS IN CULTURE

MATERIAL AND METHODS (Chapter 1)

1. Source of cells. Primary cultures of heart cells from 12-day chick embryos were prepared by the procedure described in Chapter 2. In addition, two established cell-lines supplied by the Institute of Genetics at Lund University were utilized in this study. One originated from Ehrlich mouse ascites tumour cells, established by K. Nielsén; the other was established from Chinese hamster's lung tissue in the cancer-chromosome laboratory. For simplicity, these two cell lines are referred to as "Ehrlich cells" and "Chinese-hamster cells". The culturing method for the established cell-lines was as follows: The 10 ml original medium (20% newborn calf serum + 80% Eagle MEM.) was removed from the glass flasks and replaced by 10 ml of 0.1% trypsin (Difco 1.250) in Ca^{2+} and Mg^{2+} free Tyrode's BSS (75). The trypsinization was carried out for 10 min on a shaking device (200 shakes/min). This process freed the cells from the glass surface. After some 10 pipettings by means of a 2 ml Pasteur pipette, the cells were centrifuged 1000 rev/min for 5 min. The supernatant was discarded, and the cells were resuspended in Ca^{2+} and Mg^{2+} free Tyrode's BSS and counted, and 2 ml of the cell suspension (approx. 5×10^5 cells/dish) was explanted in duplicate in plastic Petri dishes (Falcon, 35 x 10 mm) and left undisturbed for 15-20 min. During this period, all the cells settled to the plastic surface. Thereafter, the Ca^{2+} and Mg^{2+} free Tyrode's BSS was replaced by 2 ml of 30% serum samples to be tested supplemented with 70% Tyrode's BSS and left in an incubator at 37°C with a continuous gasflow (5% CO_2 in air). Growth and other parameters (see paragraph 5) were followed up to 4 days (without changing the medium) by daily observations and counting the same 8-10 fields in each dish under an inverse phase-contrast microscope. At the end of this period, the heart cell cultures were fixed and stained (see Chapter 2) and the percentage of differentiated cells was evaluated. Each experiment was performed twice in duplicate cultures. The results were highly reproducible.

2. Dialysis procedure. 4 ml of each serum sample was diluted to 10 ml by Tyrode's BSS and dialysed in a negative pressure dialysis in collodium bags^{x)} against distilled water at $+4^{\circ}\text{C}$ until approximately the original

x) Sartorius Membranfilter G mb H, SM 13200.

volume (4 ml) was obtained. The exact final volume was adjusted to 4 ml by the addition of distilled water. To make up a solution of 30% serum, the 4 ml dialysed serum was brought up to a final volume of 13.3 ml by the addition of Tyrode's BSS in a concentration, $1.43 \times$ the ordinary, that compensated for the serum salt lost during dialysis (i.e. $13.3 \cdot 1 = 9.3 \cdot X$ and $X = 1.43$).

3. Electrophoresis procedure. An adaptation of the principles of protein and lipoprotein agarose gel electrophoresis, as described by Johansson, B.G. (48), was used. However, the method was modified to some extent for serum proteins. Serum lipoprotein electrophoresis was performed according to the routine technique used in this institute.

(A) Serum protein electrophoresis. 4 μ l of each sample diluted 1:1 (50%) with Tyrode's BSS was applied to the slits (9 x 0.4 mm) of a glass plate covered with 1.5% (W/V) agarose^x). The electrophoretic separation was run with a potential gradient of 15 V/cm, for 60 min in pH 8.6 at +10°C. The proteins were fixed for 60 min in a solution of 2% acetic acid; the gel was then dried. The proteins were stained with a solution of 0.1% Amido Black B (0.1 gr A.B. + 0.61 gr Na-acetate in a solution of 87 ml dist.water + 2.7 ml glacial acetic acid and 10 ml glycerol). The plate was freed from excess dye by washing three times for 2-3 min in dye-free solution.

The electrophoretic separation of EE was performed by the same method, except that 10 μ l samples without dilution were applied to slits of 1.0 x 14 mm. For electrophoretic separation of the mixture of serum and EE, 4 μ l of the following mixture was used: 50% serum (diluted 1:1) + 20% EE.

(B) Serum lipoprotein separation. 10 μ l of each sample diluted 1:1 (50%) with Tyrode's BSS was applied to the slits (1.0 x 14 mm). The separation was performed with a potential gradient of 10 V/4 cm for 3 hours in room temperature. The lipoproteins were fixed in 2% acetic acid for 60 min. After

x) L'Industrie Biologique Francaise, Gennevilliers, France.

the gel had been dried, the lipoproteins were stained with Sudan Black (0.1% Sudan Black in 60% ethanol) for 2 hours. The excess of dye was removed by washing in 50% ethanol for a few minutes. Each sample was analysed at least twice. The preparations, after electrophoresis and staining, were read in a Chromoscan¹⁾.

4. Source of sera. The 13 types of sera used in this study are as follows:

- 1) Human, freshly obtained from one adult
- 2) Rat, pool of several adults
- 3) Rabbit, " " " "
- 4) Gerbil, " " " "
- 5) Calf, newborn (n.b.), commercial²⁾
- 6) Horse, from one adult animal, commercial³⁾
- 7) Calf, 2 month old, from one animal
- 8) Cock, from one adult animal
- 9) Swine fetus 25-27 cm, pool of several
- 10) Swine, newborn, pool of several
- 11) Swine, 1.5 days old, pool of several
- 12) Swine, 3 days old, pool of several
- 13) Swine adult, from one animal

In addition, horse sera²⁾ from 16 different horses were the object of a separate study.

Sera were prepared (except No 5 and 6) by the sterile collection of blood from the above-mentioned sources. The samples were stored at +4°C overnight, and the exuded sera were removed after the samples were centrifuged at 3000 rev/min for 30 min. Each serum was divided in aliquots of 5-10 ml and stored at -20°C until use.

As no original newborn swine serum (No 10) was available, no tests for chicken heart cells in dialysed serum, for Chinese hamster cells in normal and dialysed serum, and for Ehrlich cells in dialysed serum were carried out.

1) Joyce, Loeb1. and Co. Ltd. England.

2) Flow laboratories.

3) Statens Bakteriologiska Lab. Stockholm.

5. Evaluation of cultured cells properties. The following parameters for each cell type were evaluated.

- 1) Heart cells. Plating efficiency¹⁾ (Fig. I); % of stretched cells/dish after 24 h (Table 1); proliferation (Fig. I); degree of beating²⁾ (Table 1); % of differentiated cells/dish at the end of the culture period (Table 1); general symptoms (G. Symp), e.g., fattening³⁾, shrinkage; good cells⁴⁾ (Table 1).
- 2) Chinese hamster cells. Plating efficiency; % of stretched cells/dish after 24 h, proliferation; general symptoms.
- 3) Ehrlich cells⁵⁾. Plating efficiency; proliferation; general symptoms. Fig. I and Table 1 for heart cells, correspond also to Chinese hamster and Ehrlich cells.

1) Plating efficiency (75) = ratio of cells survived after 24 h (0-day) to cells inoculated (approx. 10^5 /dish, indicated by full circles).

2) See footnote page 95.

3) Degree of fattening was shown by arbitrary units from 0-10; for instance, + 10 indicates cells where fat droplets occupy all the cytoplasmic space.

4) Good cells indicate healthy cells, i.e. neither fattened nor yellowish.

5) Ehrlich cells were round-shaped cells that did not stretch in culture.

RESULTS

A. Behaviour of cells in normal (not dialysed) sera.

An overall look at the results after culturing different types of the cells in normal (not dialysed) sera (Fig. I) reveals that, in the first place, the survival and the growth of the cells do not correlate with the type of serum used. For instance, whereas one type of cell can survive and grow in a particular serum, survival and growth of another type of cell are inhibited in the same serum (Fig. I - 2 a, b, c; 8 a, b, c; 12 a, b, c). But it is easy to observe that, for most of the cases, different cell types exhibit similar tendency in one or other direction in a common serum (Fig. I - 1, 3, 4, 5, 6, 7, 9, 10, 11). However, it is possible to put the normal sera into the following three categories:

1. Unfavourable or toxic sera, in which the cells survive at the most 2 days (Fig. I - 1 a, b, c; 2 a; 3 a) (in this category serum No. 13 "swine adult" is also included; this was tested separately, but is not shown diagrammatically. However, the results of electrophoretic analysis are shown in Fig. II and Table 2) or survive but gradually decline, (Fig. I - 3 b, c; 4 a, b, c; 5 b, c; 8 c; 12 a, b) during the experimental period. Two major symptoms accompany the deterioration and the death of the cells, i.e. shrinkage and fattening (Table 1). Shrinkage is the dominant symptom of cell death during the initial stages of culture, and fattening mainly characterizes mal-conditioned declining cells during and at the end of the culture period.
2. Indifferent sera in which the cells survive, but neither grow nor decline (Fig. I - 5 a; 6 a; 7 b, c; 9 a, b; 12 c).
3. Favourable or promoting sera in which the cells survive and proliferate (Fig. I - 2 b, c; 6 b, c; 7 a; 8 a, b; 9 c; 10 a, c; 11 a, b, c). Evaluation of parameters other than growth reveals that very significant differences exist among the sera to support or fail to support special properties of the cells, e.g., as Table 1 shows, the stretching of the cells in normal sera varied from 0% to 100%; also the beating of the heart cells was affected by different sera. The support for differentiation (of heart cells) too was variable. Whereas some normal sera allow maximum differentiation (Table 1 - 10), in other sera, the cells exhibited partial differentiation (Table 1 - 6, 7, 8), and in still other sera, no heart cell could be differentiated. Moreover,

the plating efficiency of different cells in normal sera also varied considerably, for instance, from 0% (Fig. I - 1 b, c) to 100% (Fig. I - 6 a; 7 a; 8 a; 9 a).

B. Effects of dialysed sera on cell behaviour

A general and significant conclusion drawn from the results obtained after cultivating the cells in dialysed sera is that dialysis moderates the action of both toxic and promoting sera. The effects of dialysis being:

1. elimination of inhibitory factors from unfavourable sera, endowing them with slight improvement (Fig. I - 1 a; 2 a; 3 a, c; 4 a, b; 5 c; 8 c; 12 a, b) or even rendering them growth-promoting (Fig. I - 1 b, c; 3 b; 5 b, c).
2. elimination of growth-promoting factors from promoting sera and rendering them indifferent (Fig. I - 7 a; 8 a, b; 9 c) or even unfavourable for growth (Fig. I - 2 b; 6 b, c; 11 a, b). The latter trends can be well demonstrated by cock serum (Fig. I - 8 a, b, c) in which the shift after dialysis is highly pronounced.
3. to make the indifferent sera, promoting (Fig. I - 7 b, c), unfavourable (Fig. I - 6 a; 9 a, b; 12 c), or unchanged (Fig. I - 5 a).

These results suggest that, primarily, both growth-promoting and growth-inhibiting factors are present in serum dialysate. As Table 1 shows, dialysis of the serum also affects the differentiation of heart cells. In sera that in normal state promoted differentiation, the percentage of differentiated cells was reduced after dialysis, depending on how dialysis affected the growth of the cells. In those sera where decline followed the dialysis (Fig. I - 6 a; 9 a), the differentiation was reduced almost entirely; but differentiation was to a lesser extent affected, provided that the serum secured physical existence of the cells (Table 1 and Fig. I - 7 a; 8 a). In view of the spontaneous contraction of the heart cells, no remarkable difference was observed between cells grown in normal or in dialysed sera (Table 1). Moreover, if the cells survived during the first 24 h, generally the plating efficiency was little affected or even slightly improved after dialysis (Fig. I). However, the effect of dialysis on

stretching of the heart and Chinese hamster cells was variable. In different instances, dialysis improved, impaired, or had no effect on cell flattening (Table 1). These findings suggest that whereas the undialysable serum fraction might play the main role for survival of the cells, the serum dialysate has not a substantial effect on cell attachment, stretching, and beating. This presumption that the primary effect of the macromolecular fraction of serum refers to the physical conditions for survival of the cells was further strengthened by performing additional experiments (utilizing heart cells) in which supplementation of serum by 2.5% - 20% embryonic extract or 70% synthetic media, such as Eagle (MEM), NCTC 135, M 199 (75), did not improve cellular attachment or flattening. Fig. IX shows that, by the addition of EE, no interaction occurs between the HMW fraction of EE and serum macromolecular fraction; this suggests that EE mostly consists of substances with lower molecular weight than that of albumin (69.000 (90)). Substitution of whole serum by various concentrations, i.e. 20%, 40% and 100%, of EE resulted in the attachment and stretching of the cells, but lysis and death of the cells occurred within 4 to 24 h depending on the concentration of EE (Fig. VII). Figure X shows that the EE also contains a minute amount of HMW protein fraction that might support physical existence of the heart cells for a short period, but this fraction is not necessarily identical or similar to the macromolecular fraction of the serum. The dissimilarity is shown by the fact that:

- 1) these two fractions are not reactive and accumulative (Fig. IX);
- 2) by denaturation in 56°C for 30 min, most of HMW fraction of EE is coagulated (Fig. X). This denaturation treatment has no remarkable effect on the serum (Fig. VIII a), on the contrary, it renders the serum more stable (see page 19).

The macromolecular fraction of serum left after dialysis, besides its action in supporting the survival of the cells, in some instances (together with a simple salt solution such as Tyrode) supported multiplication of cells (Fig. I - 1 b, c; 3 b; 5 b, c; 7 b, c) without any supplementation of synthetic medium or other source of LMW organic substance. This phenomenon was directly related to the nature of the cultivated cells. Whereas established cell lines, such as Chinese hamster and Ehrlich cells, were able to proliferate in the presence of the macromolecular fraction only, the primary culture of heart cells, to behave similarly, also needed

the serum dialysate in addition to the macromolecular fraction. The action of some particular LMW substances on the multiplication of primary heart cells was further demonstrated. Thus substitution of Tyrode's BSS by 70% medium M 199 (75) considerably enhanced multiplication of heart cells (Fig. VII) when added to 30% dialysed calf serum. In some cases when, after dialysis, the growth was still in a steady state, the dialysed sera also supported differentiation (Table 1 - 7 a; 8 a).

C. Electrophoretic analysis of sera. Effects of various serum fractions on cell behaviour.

After the effect of serum dialysate was evaluated and it was shown (Fig. I - 1 a; 2 a; 3 a; 4 a, b, c; 5 a; 12 a, b) that the dialysis of some toxic sera did not reduce maximum toxicity, additional information provided through electrophoretic analysis of the serum macromolecular fraction could complete the knowledge and establish possible correlation between the behaviour of cultured cells and the quality of the serum as a whole. Fig. II presents the results. The interpretation of the findings of electrophoresis was rather difficult. Thus, although the relative positions of albumin and γ (gamma)-globulin are known, it was difficult to establish the location of other globulins, i.e. α (alpha) and β (beta) etc. for different species of animals. Therefore the fraction from albumin to γ -globulin was analysed as one unit. Concerning the lipoprotein fractions of the sera and their effect on the cells, no quantitative correlation could be found between lipoprotein electrophoretic pattern as revealed by the method used in this study, and the behaviour of the cells in culture (Fig. I and XI).

1) Effect of serum albumin fraction

The results obtained (Fig. I, II, and Table 2) clearly show that a high level of albumin in serum makes it toxic. With one partial exception (Fig. I - 2 b, c), toxicity was thus found to be associated with sera in which the albumin fraction amounts to more than 40% of the total protein content of the particular serum (Fig. IV and Table 2). This tendency is even more pronounced in view of the absolute amount of albumin (Fig. V and Table 2), i.e. when the quantity of albumin exceeds a certain level

in any sample it causes toxicity. Fig. V shows that when the albumin levels in some samples exceed the lowest level by at least 6 times, the serum becomes toxic (except No. 2 b and c). On the other hand, serum No. 12 has a rather low level of albumin (Fig. II, IV, V, and Table 2) and still it exerts partial toxic effects (Fig. I - 12 b, c). None the less, the exceptional behaviour of the two mentioned sera (2 and 12) might be explained as follows: 1) The relatively high quantity of albumin in rat serum (No. 2) just exceeds the nontolerable level (Fig. IV and V) so that the primary heart cell cultures could not stand it, but it was tolerable for the established cell lines; furthermore, as is shown later, the substances associated with albumin are the factors causing toxicity. It might be that their concentration was somewhat lower in rat serum. 2) In the case of serum 12, having a rather low level of albumin and still being toxic, the toxicity might not be associated with the albumin fraction, but might be caused by other factor(s). Thus, even if a quantitatively high level of albumin in serum often brings about a toxic effect on cell cultures, it still does not solely account for it.

To discover whether the toxicity exerted by albumin fraction is due to the "albumin-protein" per se or by the factors associated with it (90), several experiments using hearts cells from 5-, 12⁺, and 18-day embryos were performed in which an extra quantity of 16 mg/ml pure bovine-albumin¹⁾ was added to the ordinary culture's basal medium, resulting in an addition of protein approximately equivalent to that of 23% solution of whole serum (84). The results indicated no toxicity. On the contrary, this addition markedly improved cellular function²⁾ and differentiation. Thus it seems that the toxicity is related to the substances associated with albumin, and their relatively high concentrations apparently determine the toxic behaviour of the albumin fraction.

2. Effect of macroglobulins fraction

Present observations suggested that the effect of the macroglobulin fraction would be opposite to that of the albumin. Indeed, the analysis of this fraction revealed that the promoting activity of the sera was associated with these protein fractions. The separation of macroglobulin

1) Sigma, Cahn fraction V, A-4503.

2) beating

fraction into two parts, 1) γ -globulins and 2) the fraction from albumin to γ -globulins (namely α - and β -proteins), and the analysis of the effects of these fractions gave the following results:

- a) γ -globulins. As far as absolute values (Table 2 - column 6 and 7) are concerned, no correlation existed between the quantitative levels of γ -globulins and the behaviour of the cells (Fig. I and Fig. II). In some cases, it could clearly be seen that relatively high (Table 2, sera No. 5, 6 and 11), low (sera No. 9 and 10) and intermediate levels (sera No. 7 and 8) of γ -globulins had the same promoting effect, whereas in other cases, high (serum No. 13) and low (sera No 1, 2, 3, and 4) and intermediate level (serum No. 12) of γ -globulins also had the same toxic effect. Regarding the relative amounts of γ -globulins (Table 2, column 8) in the same serum, there appeared a tendency in which sera relatively rich in γ -globulins had promoting effect (Table 2, sera No. 5, 6, 8, 9, 10, 11) and sera relatively poor in γ -globulins (1, 2, 3, 4, 12) were found to be unfavourable.
- b) $\alpha+\beta$ fraction. The main part of the macroglobulins consists of the $\alpha+\beta$ fraction, which principally determines the promoting quality of the serum. Fig. IV indicates that a serum whose total amount of globulins is less than 60% must be considered unfavourable, as obviously the rest is albumin. Elimination of γ -globulin from total globulins actually renders most of the sera impotent. However, the level of γ -globulin might not have any significant effect on the quality of the serum as regards absolute quantities, but the crucial contribution of γ -globulin seems to be its increase in the level of the total globulins in the serum relative to albumin fraction. Fig. IV well demonstrates this trend. Without the addition of γ -globulins, the sera No. 7, 8, 5, 6, 11 would be ineffective. The effect of albumin/globulin (A/G) ratio is also demonstrated (Table 2, column 15). Values from a maximum of 1.17 to a minimum of 0.16 of this ratio could be observed, and with a smaller ratio, a higher promoting action was noted.

Further analysis of the serum electrophoretic pattern revealed two additional possible correlations between composition of the macromolecular fraction and the behaviour of the cells in culture on a dialysed serum. First, a serum can be classified as favourable when its total protein content is relatively low (Table 2: column 1, 2 - sera No. 7, 8, 9, 10, 11);

conversely, when the total protein content is high, the serum can be considered unfavourable (sera No. 1, 2, 3, 4, 13). In this respect, at the first appearance, there are exceptions, for instance, serum 5 and 6, both of which are promoting sera, have a relatively high level of total protein and serum 1, a toxic serum, actually has a rather low total protein content. Observations further reveal that for serum 5 and 6 the high total protein content is contributed mainly by macromolecular fractions other than albumin (Table 2, column 14). For serum 1, the total protein content is relatively low, but still 54% of it is contributed by albumin fraction (Table 2, column 5). Accordingly, the quantitative toxicity of serum macromolecular fraction is mostly associated with its absolute and relative amount of albumin.

Second, the complexity of serum macromolecular composition seems to play a significant role for the behaviour of cells concerning their attachment and maintenance in culture. Sera having a complex electrophoretic pattern, i.e. displaying a very heterogenous composition visualized as multiple peaks in electrophoretic pattern, can apparently be classified as toxic sera (Fig. II, sera No. 3, 12, 13). This qualitative phenomenon can explain the exceptional character of serum 12, i.e. being toxic despite having low albumin and low total protein.

Finally, all the above-discussed parameters of dialysed serum can be well demonstrated in the series of prenatal, newborn, and post-natal swine sera (Fig. I, Fig. II and Table 2, sera No. 9, 10, 11, 12, 13). It is seen that serum-albumin level, total protein content, and electrophoretic complexity increase with increasing age of the animal. Parallel to this increase, the ability of serum to support the survival of the cells decreases

D. Effect of individual, pooled, and heat denatured sera on the behaviour of heart cells.

1) Varying effects of individual and pooled sera

The 16 individual normal, not denatured, horse sera studied exhibited large variations in support of heart cells to survive and express their characteristic properties. Although taken from the same species, the investigated individual sera ranged from rather toxic (a few) in which the cells

gradually declined, to favourable (most) in which the cells survived and expressed their characteristics in large variations. For instance, after 24-hour culturing in the latter sera, the plating efficiency (attachment) varied between 30 and 90% (mostly around 70%) and the stretching ranged between 20 and 90%. The spontaneous contraction, growth, and differentiation also varied considerably in different normal horse sera. The results somewhat resembled previous results with sera from different species, suggesting that the behaviour of cells in any type of serum depends primarily on the quality of the serum sample involved. Pooled sera from 4-6 animals given to the cells did not cause any improvement in the state of cultured cells; on the contrary, the cells in such pooled sera became more susceptible to the culture conditions. It could be noted that in some very rare instances, when a satisfactory serum was mixed with EE, it became unfavourable for growth of the cells, despite this mixture usually constituting ordinary growth medium. This toxic effect was frequently observed when pooled sera was mixed with EE. Analysis of electrophoretic pattern of mixed HS + EE reveals that no interaction occurs between the EE and macromolecular fraction of the serum (Fig. IX). Therefore it seems that interaction has taken place between the LMW fraction of EE and serum.

2) Effect of heat denaturation of serum

In general, the effect of horse serum, denatured at 56°C for 30 min, was a stabilization of the growth of heart cells, whereas in normal sera, the growth was variable and occasionally decline occurred. By growing heart cells in denatured serum the decline effect was diminished (Fig. VI) and was replaced by steady state or slight growth.

Simultaneously, the ability of heart cells to differentiate increased markedly in denatured serum. Other parameters, such as attachment, stretching, and beating, were unaffected or slightly favoured. After denaturation, the susceptibility of heart cells in pooled sera solely, or mixed with EE, was decreased too. The avoidance of decline is by itself a condition for the cells to express their properties.

For further verification of the effect of denaturation, sera from horse and also from newborn and adult calf, all of which previously showed

varied effect on the growth of heart cells, were tested as both normal and after heat-denaturation at 56°C for 30 min. In addition, the denaturation was extended to 64°C for 12 min (before coagulation started). The results given in Fig VI show that, by increasing the denaturation temperature, the growth stimulatory activity and also the stabilizing property of the sera increase. Other parameters, such as attachment, stretching, and beating, were unaffected by this treatment.

When finally H.S. was denatured at 80°C for 2.5 min, more than half of its macromolecular fraction was coagulated (Fig. VIII c). The supernatant after centrifugation (3000 rev/min, 30 min.) was incorporated in the culture medium at the usual 30% concentration and was found to support cell attachment, stretching, growth, and pulsating activity just as well as serum denatured at 56°C for 30 min (Fig. VIII a).

3) What kind of changes occur in the serum macromolecular fraction by heat inactivation^{x)}

Comparison of the serum protein electrophoretic pattern after heat denaturation at 56°C for 30 min. with normal serum revealed the following (Fig. VIII a and Table 3): Through a slight increase in the electrophoretic mobility of albumin, the α_1 -globulin fraction, previously mostly covered by albumin, was now better distinguished. The relative amount of different fractions of the two samples was almost unchanged (Table 3, column 5 and 6). Also the absolute amount was the same, except for a slight increase in albumin, α_1 , β , and total protein in denatured serum (Table 3, column 1 and 2). The γ -globulins were not altered either qualitatively (Fig. VIII a) or quantitatively (Table 3, column 1, 2 and 5, 6).

Heat inactivation of the horse serum at 64°C for 12 min before coagulation started had more extreme effects on the serum (Fig. VIII b and Table 3). The general tendency was a greater mobility of the albumin fraction. Regarding absolute values (Table 3, column 1 and 3) there was a decrease in total protein content (14%). This decrease was contributed by loss in albumin, in α_1 , α_2 , and γ -globulins. These values also decreased in relative amount in the same serum, but to a lesser extent; e.g., for α_1 , α_2 , and γ -globulins, there was no remarkable difference between normal and denatured (64°C) serum (Table 3, column 5 and 7). The albumin decreased about 12%.

x) To identify the α and β globulins, comparison was made with human serum.

But there was a significant increase in the amount of β -globulin region, 27% in absolute and 15% in relative values, but this increased amount was diffused over the whole β -region (Fig. VIII b). The α_1 -fraction became more distinct qualitatively (Fig. VIII b) although not quantitatively (Table 3, column 5 and 7).

Partial coagulation of serum after denaturation at 80°C effected an increased electrophoretic mobility of albumin (Fig. VIII C). The absolute amount of all serum components decreased approximately 53% (Table 3, column 1 and 4). In the relative amounts of different fractions (Table 3, column 5 and 8), there was a decrease in albumin, whereas the proteins of the α -, β - and γ -region were not distinct and behaved as a continuous diffusible sheet of homomolecular substance (Fig. VIII c).

Concerning some gross observations on physical properties of the serum, such as coagulation, viscosity, and filterability before and after denaturation, it was found that, although these properties were unaffected when serum was denatured at 56°C for 30 min, the denaturation at 64°C for 12 min before coagulation began made the serum more viscous and more difficult to filter, despite no visible coagulation having occurred. (The serum was not centrifuged before filtration). The most remarkable change occurring in serum denatured at 80°C for 2.5 min was the coagulation of the serum proteins. After centrifugation for 30 min at 3000 rev/min, the coagulum constituted approximately 2/3 of the normal serum. The supernatant was as filterable and viscous as normal horse serum.

DISCUSSION AND CONCLUSIONS

The results clearly show that several single factors in the serum, acting individually or collectively, determine the quality of the serum as a physico-chemical "milieu" for in vitro cultivation of cells. Although no rules have been found in present investigation for the behaviour of cells in various types sera, some remarkable tendencies for behaviour of different cell types in most of the sera tested were established. Thus some factors of serum when present at a threshold level have the same common effect. This is particularly obvious when considering toxicity of a serum. Here, the simple effect of causing rapid cell death might be due to even one single factor. On the other hand, for survival and functional activity in any stage of culture, a complex of associated factors might be needed. The factors in the order of their significant influences and mode of action on the survival and functional properties of the cells have been found to be:

- a) The dialysable fraction, which contains the LMW substances that primarily determine the toxicity and the inhibitory and promoting effects.
- b) The nondialysable fraction, consisting of an albumin fraction whose relative or absolute high level turns the serum toxic and the globulins that form the crucial fraction which determine the physical survival and consequently the proper functional activities of the cells.
- c) Additional protein fractions, appearing in serum, and in electrophoretic pattern exhibited as multiple peaks. This pattern is accompanied by an increase in toxicity.
- d) The total protein content of the serum, which, if high, can be considered to be toxic when its albumin component exceeds a certain level.

Olmsted (72), by performing several physical and chemical measurements on a total of 96 fetal calf sera (FCS) and by carrying out biological tests using the individual FCS samples as a part of culture medium with MDBK, HeLa, and primary dog kidney cells, found that the chemical parameters which correlate with toxicity in FCS include a high total protein content, an abnormality in the protein electrophoretic pattern, a low acid-soluble phosphorus content, and a high phospholipid and total cholesterol content.

Furthermore, the total protein increased with increasing age of the fetus. With a different experimental system -- i.e. other cell types, other sera, and only electrophoresis as analytical method -- results of the present study agree with the above conclusions, as far as the toxicity is concerned.

However, in the present system, the obtained results provide more details through the use of a broader spectrum of sera from various animals species and different donor ages. For instance, Olmsted (72) states that, although a high protein content did correlate with toxicity in some FCS, this could not be the only factor for occasional toxicity. Also, it has been found here that high protein content per se has a positive correlation with biological toxicity, provided that relatively more than 40% of total protein is contributed by the albumin fraction. Otherwise, the serum has a promoting effect on all parameters concerned. Moreover, in this study too, it was found that in swine serum the total protein of serum increases with increasing age of the donor. This is paralleled with increased toxic affect of the serum. In this respect, Karlsson (49), analysing the sera of developing pig fetus, found that, with advancing foetal development, the albumin content increased. In addition, it was shown here that the complexity of the swine sera proteins, as shown by electrophoresis pattern, increases with postnatal increase in age and also correlates with biological toxicity. The abnormality found by Olmsted (72) -- i.e. an unusual band in the α_1 -globulin, distinct γ -globulin bands, plus three bands in the α - to β -globulin range -- and the complexity found here in electrophoretic pattern of toxic sera might reflect the same tendency. In his system too, the abnormal sera showed an increase in complexity with the addition of new electrophoretic bands. However, the basic result of the present study, i.e. the correlation between toxicity and the A/G ratio, does not agree with the results obtained by Olmsted (72), who found that the A/G ratio of 55 FCS tested samples ranged from 0.84 to 7.95 with no correlation of the A/G ratio with biological tests. The range of A/G ratio (0.16-1.17, Table 2, column 15) observed in this study does not show so wide a difference in the A/G ratio as those obtained by Olmsted, despite the samples in this study being collected from different species and donors of extreme ages. But the considerable difference in evaluating this ratio in the two systems might to some extent account for this discrepancy.

Two properties, i.e. the toxic and the growth promoting effects of the sera, were found to be associated primarily with the dialysable LMW fraction of the sera. After dialysis, both these effects were eliminated or reduced. An analysis of a variety of substances constituting this dialysate fraction would be an enormous task. Of the many authors who have sought for the growth-promoting factors in serum, few have tried to discover its toxic factors. Olmsted (72), as already mentioned, found that toxicity of the serum is correlated to its low acid soluble phosphorus, high phospholipid, and high cholesterol content.

Schultze and Herman (90) summarize the biological functions of human serum albumin as follows: 1) transport function, 2) acceptor for fatty acids in lipid metabolism, 3) regulation of plasma volume by colloid osmotic pressure, and 4) storage form of protein and amino-acids. The present study showed that serum samples with relatively high level of albumin exert toxic effects on the cells in culture. On the other hand, it was concluded that this effect results, not from albumin protein per se, but from substances associated with it, in particular, high concentrations. However, among the above-mentioned albumin functions it seems probable that the role of albumin in lipid metabolism is more connected to its toxic effect. In this connection, Baily (9), studying cellular lipid nutrition and lipid transport, found that most of the cell lipids in culture are derived from the serum. Triglycerides are most rapidly taken up and considerable quantities of phospholipids and cholesterol are removed from the medium. Accordingly, Geyer (26), studying uptake and retention of fatty acids by tissue culture cells, concluded that cells in tissue culture can take up and convert long-chain fatty acids to CO_2 , cholesterol esters, triglycerides, and phospholipids. And Moskowitz (70) argued that exogenous fatty acids were quite sufficient to account for extreme degrees of lipid accumulation in monolayer cell cultures. This uptake of lipid substances from the serum by cells, and the findings of Olmsted (72), that lipid substance in the serum correlated with biological toxicity and function of albumin in lipid metabolism, and also the phenomenon of fattening and decline and death of cells observed in the present study suggest that a high degree of correlation might exist between a serum rich in lipid substances of LMW and its toxicity.

This idea was supported by the finding that analysis of HMW lipoprotein fraction by its electrophoretic pattern, resulted in no correlation between the toxicity of the serum and its content of α and β lipoproteins. However, two clear symptoms accompanied the state of the cells in the unfavourable sera: a) shrinkage which dominated cell death at the initial stages of culturing period, and b) fattening that characterized deteriorating cells during and towards the end of the culture period. (In Ehrlich cells, fattening was not clearly visible; thus the shrinkage symptom dominated). The very interesting fact observed was that, by dialysis, the fattening did not disappear, but in some cases replaced the shrinkage symptom of the predialysis (Table 1). The shrinkage symptom observed in this study does not correlate with the function of albumin as a regulator of osmotic pressure, as addition of extra quantities of albumin did not increase cell shrinkage; on the other hand cells in sera No. 8 and 9, having relatively low albumin levels (Table 2), also exhibited this symptom (Table 1). As the fattening effect continues to exist in toxic sera despite the dialysis, and as it is apparently not quantitatively contributed by the HMW lipoprotein fraction, this suggests that dialysis might not effectively remove the LMW lipids, a likely presumption, as will be seen later. On the other hand, Rinaldini and Dick (84), culturing explants of heart in Tyrode's BSS supplemented with 14 mg/ml crystalline serum albumin (equivalent to 20% serum), found that, after 10 hours, some cells began to show vacuoles and abundant lipid granules. However, as they concluded, this effect was probably the result of nutritional deficiency. Present results show that fattening also occurs in sera relatively poor in albumin, i.e. serum No. 11 and 12 in which the toxicity was found to be correlated with serum protein heterogeneity. Bensch et al. (10) found that L cells accumulate lipid from the extracellular medium and as a result of de novo synthesis and concurrent with cessation of growth (protein synthesis). Grunbaum et al. (10) state that one of the first symptoms of deterioration of tissues cultured in vitro in the presence of old medium or after prolonged existence without being subcultured is the formation of fat globules in the cytoplasm. Also Mackenzie et al. (59) found that acidification of the medium of several cell lines caused a striking increase in the number of cytoplasmic perinuclear granules, identified as lipid-rich particles. As generally recognized, fattening is a usual phenomenon in senescence and death of cells. The conclusion from the above findings suggests that the accumulation of fat granules in the cytoplasm, in addition to the uptake from external sources (e.g. serum), is a response displayed by mal-conditioned cells, caused by a variety of factors.

On the other hand, the peptides, amino acids, and other LMW substances, presumably split products of proteins, are probably growth-promoting components present in dialyzable fraction. According to Harris (37), cultures of freshly isolated chick skeletal muscle fibroblast, in 10% dialysed chicken serum supplemented with 50-90% synthetic medium 199 (dial CS 199), proliferated normally for a day or two, then growth tapered off and ceased after 3-4 days. In the present study, heart cells in the presence of 30% dial CS + 70% M 199 proliferated normally for 4 days. The deficiency syndrome described by Harris could be prevented if serum dialysate was incorporated in the initial dial CS 199 medium. The addition of 0.1% proteose-peptone to the dial CS 199 might, according to Harris, substitute for the serum dialysate and restore full activity to whole serum. The effects of proteose-peptone and tryptose phosphate suggest that the missing factor might include peptides or other split products of protein cleavage.

Amborski et al. (6) point out that LMW materials separated by gel filtration from tissue hydrolysate can replace the serum for growth of some established cell lines in vitro and primary cells cultures (Amborski et al. 7). Moreover, they add that the active material appears to be a mixture of products, possibly peptide in nature, with molecular weight between 700 and 1600. Similar results were obtained by Michl (65), who found that the addition of lactalbumin hydrolysate to a medium consisting of α -globulin fraction plus albumin and a synthetic medium accelerated the growth rate. Leiberman et al (57, 58) also found that, for the multiplication of cells, in addition to a purified serum fraction (α -globulin), albumin and some heat-stable dialysable compounds present in peptons are required.

Functional activity was, in the present investigation, shown by heart cells in both dialysed and normal sera, suggesting that the macromolecular fraction supplemented with the Tyrode's simple BSS is sufficient to allow spontaneous contraction of the heart cells. These results might support the observations of DeHaan (20) that K^+ ion concentration reversibly regulates the spontaneous activity of the heart cells. Harary et al. (34), extracting the total lipids of the serum, found that heart cells incubated in lipid deficient media lose their ability to beat, but the addition of serum lipids or fatty acids to the cells restores this ability. Application of these results to those obtained here after dialysis reveals that

1) the albumin and the lipoprotein fractions might represent a carrier and a source of lipids, respectively (90), for this purpose and 2) dialysis as mentioned earlier does not remove all the LMW lipids from the serum.

Another unexpected observation was that, in some samples after dialysis, an active proliferation of cells took place (Fig. I - 1 b, c; 3 b; 5 b, c; 7 b, c). It means that dialysis not only reduced growth inhibitory factors from undialysed sera in these cases, but also promoted cell multiplication. This seems rather paradoxal, as it was previously found that the growth-promoting factors were primarily associated with the LMW substances eliminated with the dialysate. However, it was revealed that this depends mainly on the origin of the cell, i.e. whereas the primary heart cells could not grow in dialysed conditions, the established cell lines, in some samples, proliferated actively under the same conditions.

Results obtained by Harris (37), when cultivating primary skeletal muscle cells, and Eagle (as cited by Harris, in 37) culturing several established cell lines in dialysed serum, support the above observations. Most probably, dialysis does not eliminate from the serum all the LMW growth-promoting, bound substances, e.g., peptides and amino-acids, cofactors needed for the proliferation mechanism. It was previously supposed that fatty acids required for the beating function are present in the albumin-fatty acid complex. Fisher et al. (25) gave further evidence in the literature in favour of this. They stated that two protein constituents of the serum play an active role in cell growth in vitro: a) albumin which functions, at least partly, as a carrier of essential small molecules, and b) an α -globulin. Pierson et al. (77) considered that, in whole serum, small active compounds are probably bound to larger carrier protein molecules or exist aggregated. Eagle et al. (22) suggest that the serum protein serves primarily as a carrier for essential growth factors.

Although we have not demonstrated active uptake of HMW serum proteins by an analytical method, the proliferation of cells in certain dialysed samples suggests the utilization of macromolecular fractions for this purpose. For instance, sample 1 b and 7 b (Fig. I), during the first two days of exponential growth phase, were successfully duplicated 2 and 2.3 times, respectively. The reports in the literature mainly support the idea of

utilization of serum proteins by cultivated cells. By electrophoresis analysis, Healy et al. (40) found a decrease in α_1 -acid glycoprotein and α_2 -macroglobulin during the cultivation of mouse embryo cells. Bachtold et al. (8) observed utilization and loss of the α_2 -globulin fraction from serum during the first days of cultivation of monkey kidney cells. Tritsch (97), growing 2 lines of cells in fetal calf serum repeatedly, found that after the second cycle of cell growth, the serum contained almost no α -globulins and only little β -globulin. And Rubin et al. (86) observed rapid depletion of α and β fractions of serum from the medium, whereas cultivated cells increased in size.

On the other hand, Eagle et al. (22) showed that, by cultivation of HeLa cells in labelled protein, only 3-6% of newly synthesized cells are labelled. Michl et al. (66), by growing HeLa cells in a labelled α -globulin, state that the protein moiety of the globulin in a synthetic medium is not substantially incorporated into the cells. However, the lack of α -globulin incorporation into the cells in Michl's experiment might be due to the supplements used, such as synthetic medium and 0.1% albumin. This supposition is further supported by the conclusion of Healy et al. (40). According to them, the degree of complexity of a basal medium to which test supplements are added determines the response of the cells to these supplements, i.e. a relatively simple basal medium makes greater demands on a protein supplement than a more complete and adequate medium.

Even after dialysis had removed its LMW substances, serum did not become an inert and indifferent substratum for survival and growth of the cells, but still continued to show one or other of its previous properties, i.e. a toxic, indifferent, or promoting effect. But in this case, the particular property was obviously related to the distinct albumin or globulin fractions. The findings demonstrated that, if the relative amount of albumin in a particular serum exceeded values above 40% or if its absolute amount exceeded a certain level at least 6 times higher than the lowest, it conferred toxic properties to the serum. This study did not reveal a quantitatively gradual effect of albumin, but a threshold effect; moreover, it did not determine the real function of albumin in optimum levels; instead, its function as a carrier and storage of some LMW substances became obvious. Regarding the effect of albumin protein per se, Rinaldini (84) found that the presence of 14 mg/ml crystalline albumin in Tyrode's BSS

prevented lysis of dissociated heart cells, but the attachment of cells did not occur. (Similar results were obtained in the present study by using from 5-25% egg albumin in Tyrode's BSS). Albumin is apparently a protection against lysis.

Philips et al. (76) suggest that, in certain HMW substances which will take water, such as gelatin, blood albumin or methyl cellulose will completely protect cells from lysis when added to a saline solution of the same concentration as that of protein in interstitial fluid.

Michl (65), attempting to isolate an active α -globulin from the calf and horse serum, also found that 0.4% albumin added to an α -globulin fraction is of essential importance for the growth of HeLa and monkey heart cells, the effect apparently being protective and nonspecific. Also it is possible to substitute some other high molecular substances for albumin, for instance, methyl cellulose or P.V.P. (polyvinylpyrrolidone).

Fisher et al. (25) found that two protein constituents played an active role in cell growth in vitro. One being albumin, which may function, at least partly, as a carrier of essential small molecules that support the nutritional state of the cells without displaying any cell stretching activity. The fraction that enabled the cells to attach to the substratum and to stretch, and consequently to promote further functional activity, was found to be the globulins fraction of the serum. In the present study, after dialysis, the latter properties, i.e. attachment and stretching, were not impaired, whereas supplementation to or substitution of this fraction by other biological compounds, such as EE, did not improve or substitute for this ability. None the less, 100% EE allowed attachment and stretching of the cells, but did not support survival for any long period. However, the addition of 16 mg/ml bovine albumin to the basal medium in the present study considerably improved cellular function and differentiation. This suggests that this addition serves some purpose or is utilized for structural and consequently functional purposes.

The gamma globulin fraction per se, in this study, was found to play an insignificant role for the behaviour of cells in culture, although its relatively high participation in total protein improved the quality of the serum. This contradicts the findings of Healy et al. (40) and Eagle (cited

in 40) that γ -globulins exerted toxic effects. When presented in relatively low quantities, γ -globulins were in some cases even found to be associated with toxic sera.

Jainchil et al. (47) found that serum deprived of its γ -globulin by alcohol precipitation supports survival of cell-lines, but not cell division; the addition of precipitate resulted in rapid resumption of cell division. They also found that the factor itself was not a gamma globulin, because natural agamma-globulins are potent in stimulating DNA synthesis, but a component that precipitates with γ -globulins. Rubin et al. (86) found no increase in cell size (increase in protein content) on cultivation on γ -globulins.

Accordingly, it was judged that the remaining part of the globulin fraction, i.e. alpha + beta globulins, should be the principle determinants of the quality of the serum, but we have not further distinguished between α and β globulins. The positive effect of the globulin fraction was preserved even when the globulin fraction was drastically reduced either by denaturation in 80°C or by lowering serum concentration from 30% to 2.5%. The same stimulus was effected even by washing the cells merely in serum, thus Rinaldini (84) observed that spreading and attachment of the dissociated heart cells in protein-free media can be achieved if the cells are previously washed with serum. He concluded that components of serum other than albumin, presumably absorbed on the cell surface, are required for their normal spreading. Similar results were obtained by Holms (43) who isolated an α_1 -protein from human serum, of which as little as 0.4 μg per ml was effective in stimulating the spreading and growth of HeLa cells. Leiberman et al. (57) also isolated a fraction that in electrophoresis moves as α -globulins, 3.0 $\mu\text{g}/\text{ml}$ being enough to cause flattening of a whole population of cells. Fisher et al. (25) demonstrated that much, if not all, of the attachment and stretching activity of the cells is associated with the macromolecular serum components. They also isolated an α -globulin from adult calf serum which promoted attachment and stretching of cells on glass surface. The fetuin system of fetal calf serum also possessed similar activity. Both fetuin and material isolated from normal serum exercise a strong antitryptic action, and the authors suggest that the mechanism of attachment and stretching might be a reflection of the ability to inhibit tryptic digestion of outer-cell-wall proteins. Weiss (102) also found

that trypsin-treated cells are associated with a loss of adhesive properties, which is restored by the addition of serum to culture. He isolated a fraction rich in lipoprotein and α -globulin that can replace the whole serum.

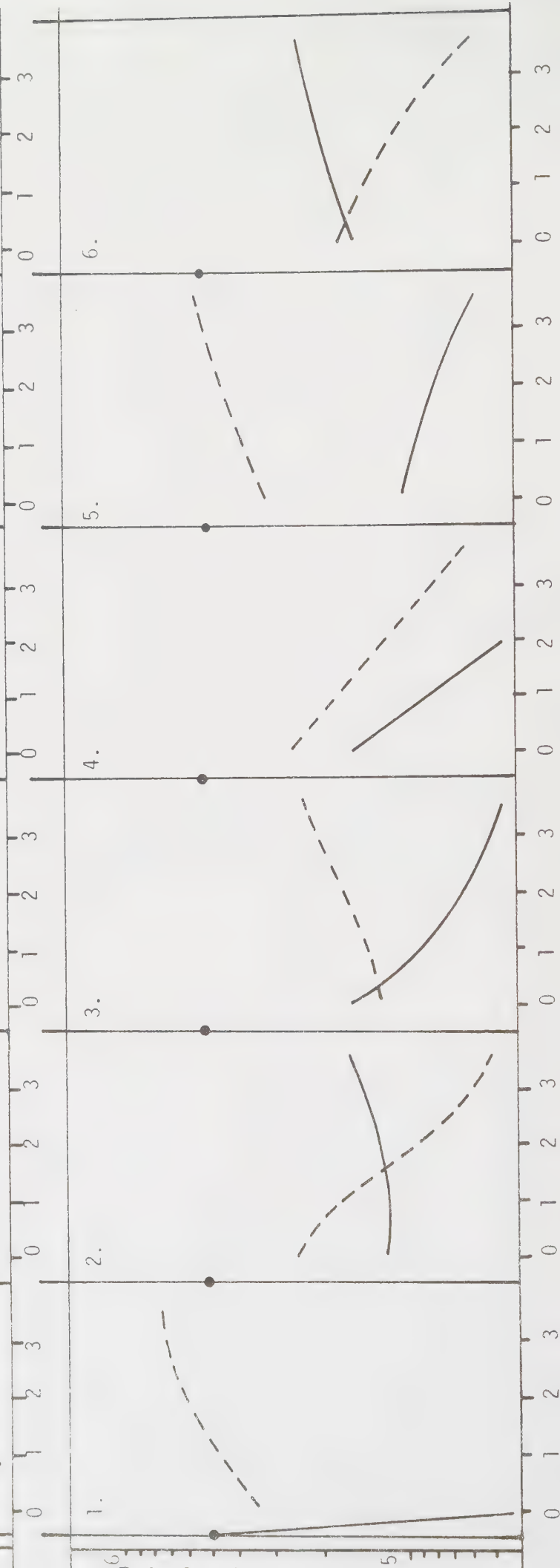
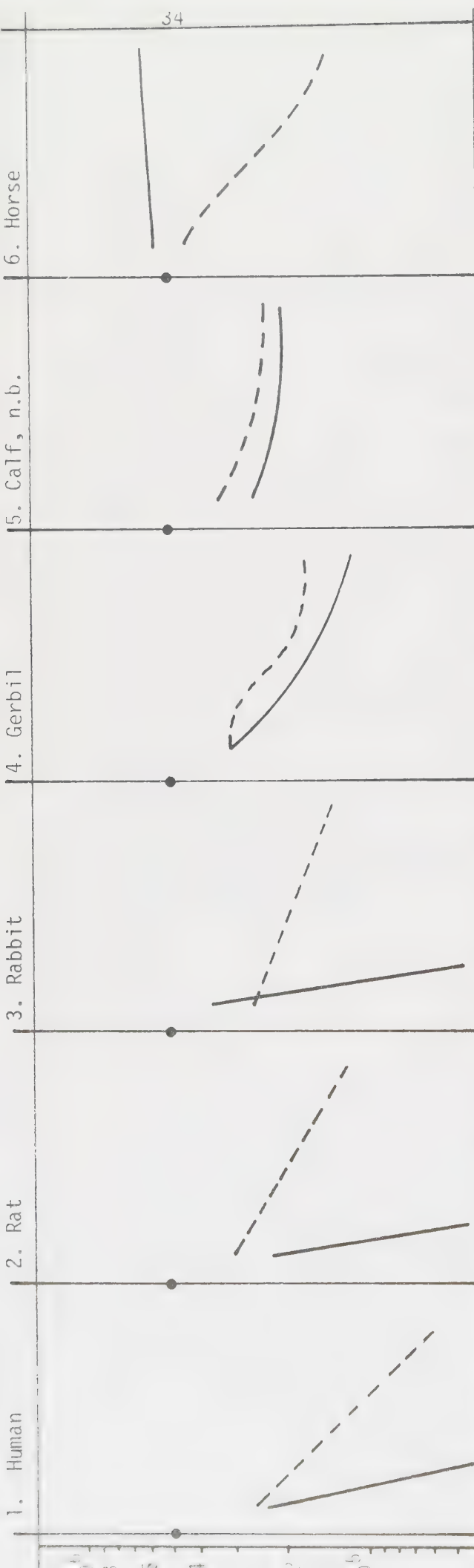
Lieberman et al. (57) purified from different sera -- i.e. calf, human, guinea pig, rabbit, rat and fetal calf -- a factor that caused cell attachment and flattening and which was mainly or entirely composed of glycoproteins. Electrophoresis of the factor revealed two components which move as α -globulins.

Similar results have been reported by Healy et al. (40) and Michl (65) who found that the globulins are the active part of the serum that causes rapid attachment of the cells and formation of monolayers; maximum specific activity was found to be associated with α -globulins. The present results, and also those cited above, suggest that the globulin fraction even in minute amount contributes a crucial function and serves the dissociated cells and cell lines for their attachment and flattening and proper function in culture. Although this fraction's mechanism of action is not very well elucidated, Fisher et al. (25) and Weiss (102) attributed the action to the antitryptic effect of this fraction. Wallis et al. (99) showed the effect to be inhibition of the action of residual trypsin after cell dissociation and of proteolytic enzymes synthesized by cells. Rubin et al. (86) suggested that serum macromolecules exert at least part of their growth-promoting effect by increasing cell permeability to required substances.

Michl et al. (67) suggested that α -globulin influences ion transport to and from HeLa cells; in another report (68), they stated that the α -globulin serves as a source of high energy phosphate group or as a suitable phosphate carrier. Furthermore, they argue that the primary function of α -globulin in the cell culture, and also in the whole organism, seems to be regulative and specific rather than nutritional or physico-chemical.

Witkowski et al. (104) suggested that the possible role of the serum on attachment of the cells to glass surface was a modification either of the cell or the glass surface; Lieberman et al. (57) speculated that the protein (α -globulin) serves as a carrier of a sugar derivative that cannot be synthesized by the mammalian cells.

As is well known, the use of serum (or its particular fraction) is required generally for cultivation of isolated cells, i.e. cells which previously have been tissue constituents, but by means of some special actions, e.g., proteolytic enzymes or mechanically, were separated from the tissue, as in primary cell-cultures, or from the substratum, as in established cell lines. But the question arose whether the function of the serum is merely the inhibition of the action of residual proteolytic (trypsin) enzymes, as suggested by some of the mentioned authors, or is simply protective, as is the intercellular material (84). The latter effect seems probable, as there are some strains of cells that, upon cultivation (after trypsin treatment), do not need the presence of serum (75), and there are reports also that even some primary cell cultures (Halle et al. 31) can be cultivated in the absence of serum, but only in defined synthetic medium. In this experiment, Halle et al. explanted the cells in a very high density concentration, i.e. $2.3-8 \cdot 10^6$ cells/0.5 ml or 1390-3900 cells/mm², the density approaching that of intact tissue, which is perhaps why explants and organ cultures do not need the presence of serum (84 and 98). Additional support for this presumption might derive from the findings of Lieberman et al. (58) who assayed a serum fraction and suggested the function of the fraction to be that of a surface protein and a polyvalent cation intimately related to the process of attachment, as many cells are readily detached from a glass surface by exposure to low concentration of either trypsin or versen.



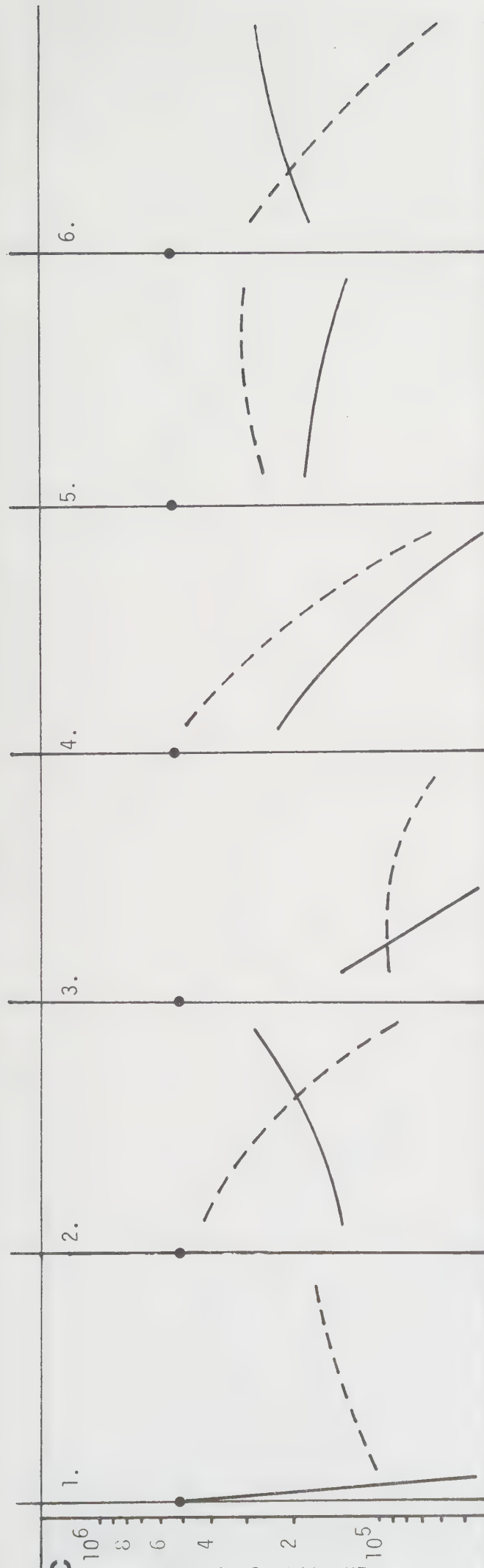
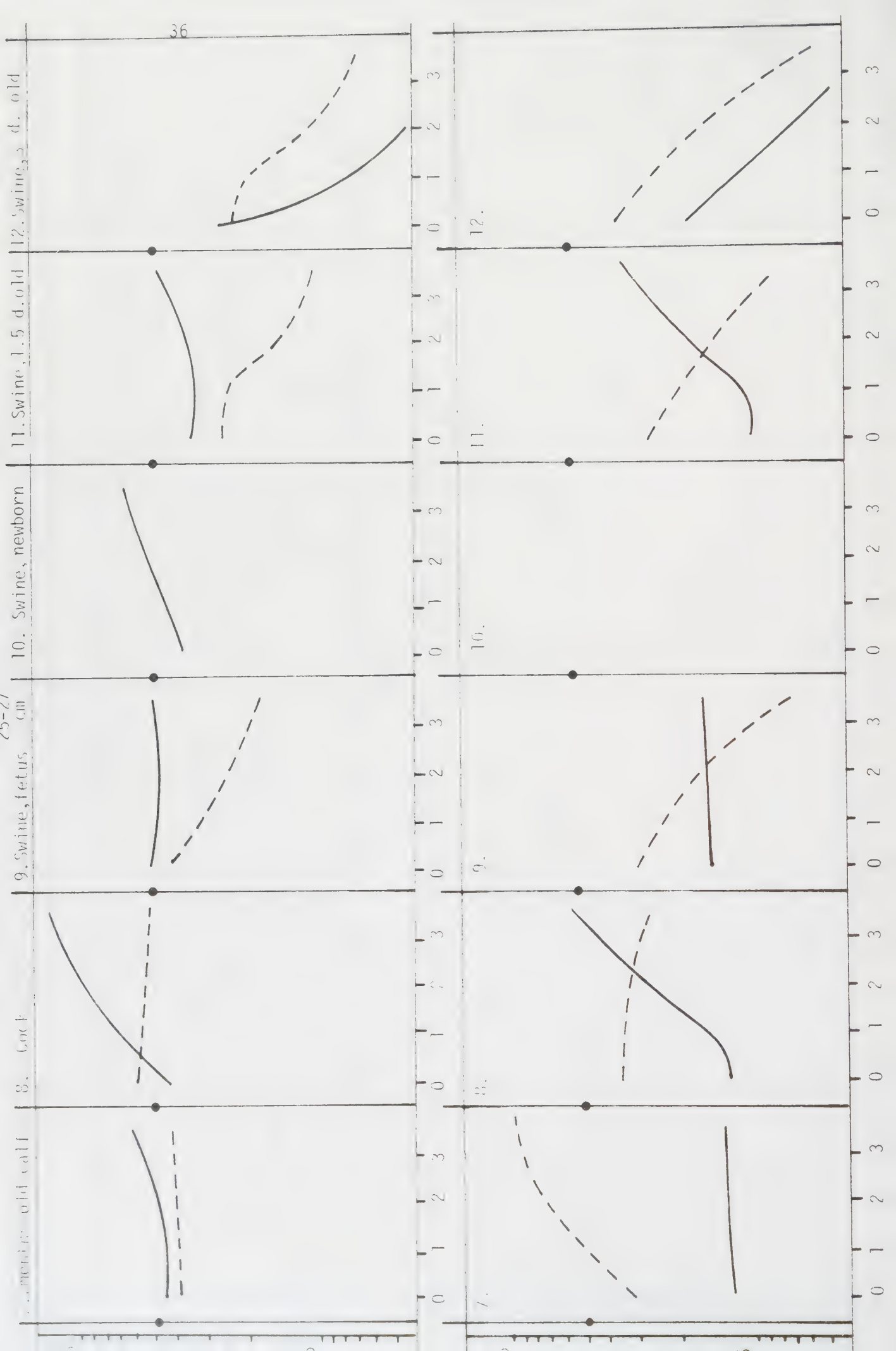


Fig. 1. Abscissa: days; ordinate: cells/dish. Behaviour of cells in normal (undialyzed) $\circ$ and in dialyzed sera $\circ$ --- $\circ$.

Table 1.												
part cells	1		2		3		4		5		6	
	not dial.	dial.	not dial.	dial.	not dial.	dial.	not dial.	dial.	not dial.	dial.	not dial.	dial.
retch	-	20	-	40	-	-	70	70	60	60	80	60
ating	-	low	-	low	-	-	low	low	low	low	high	low
d.c.	-	-	-	-	-	-	-	-	-	-	60	5
symp.	shrunk	fat + 4	shrunk	fat + 8	shrunk	-	fat + 10	fat + 8	good cells	good cells	fat + 3	fat + 3
inese cells												
retch	10	95	90	-	70	95	-	-	40	100	100	50
symp.	shrunk	good cells	good cells	fat shrunk	shrunk	good cells	fat + 8	fat + 8		good cells	good cells	shrunk
rich cells												
symp.	shrunk	good cells	good cells	good cells	shrunk	shrunk	shrunk	shrunk		good cells	good cells	shrunk



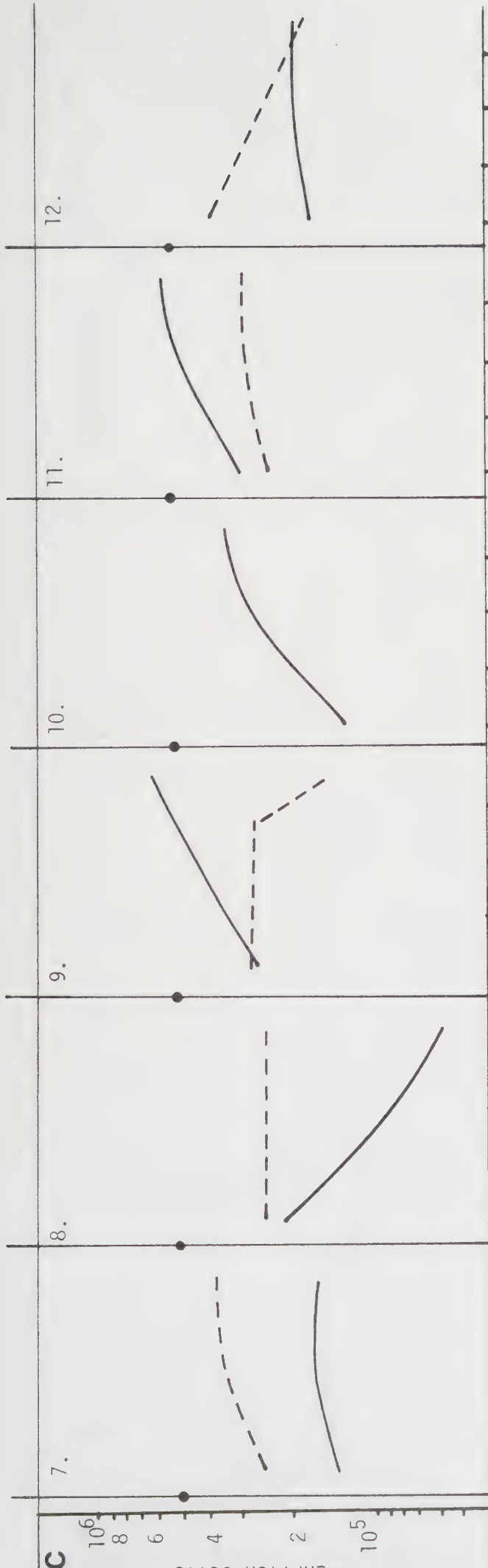
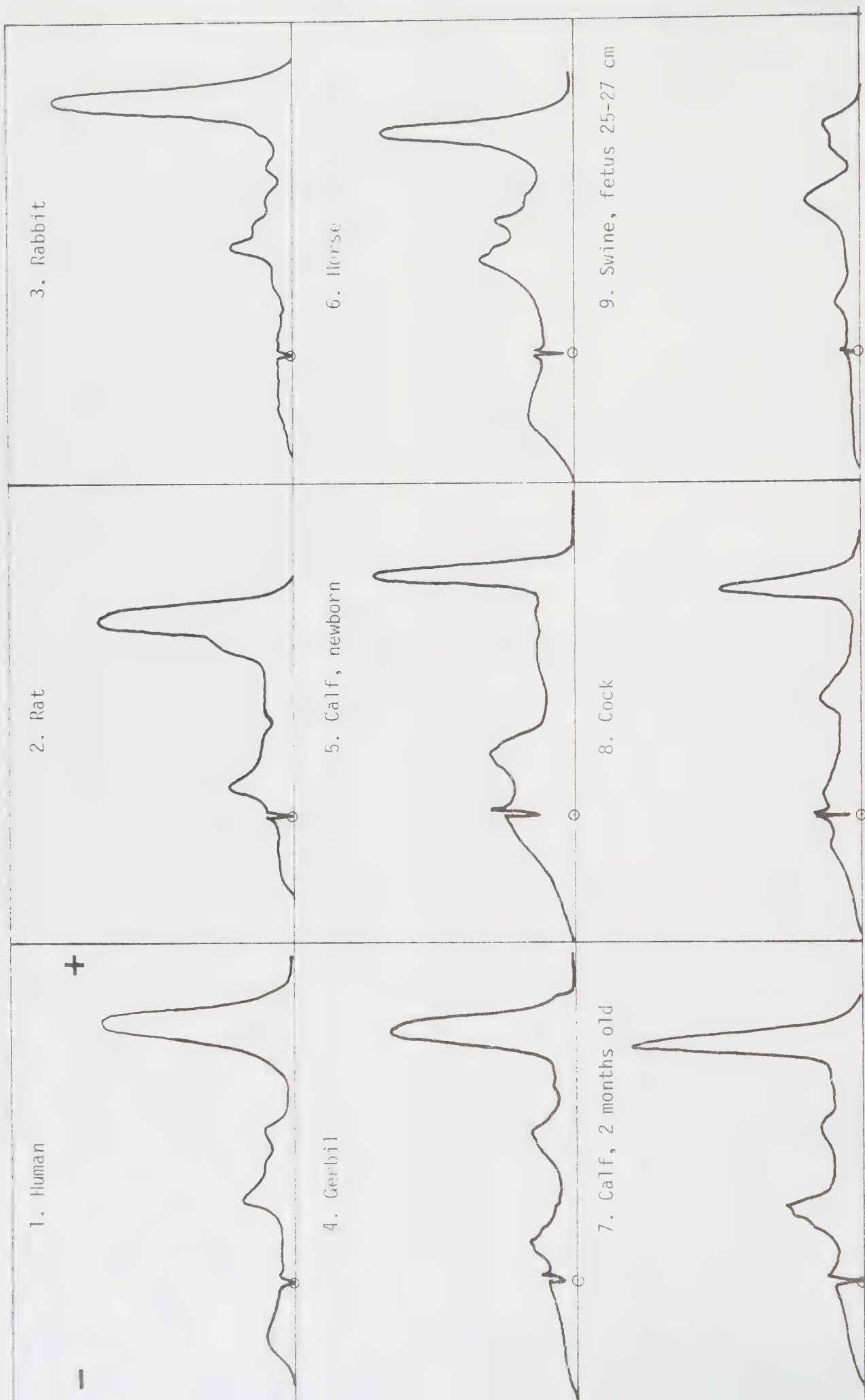


Fig. 1. Abscissa: days; ordinate: cells/dish. Behaviour of cells in normal (undialyzed) $\circ$ — $\circ$ and in dialyzed sera $\circ$ --- $\circ$.

Table 1

part	not dial.	7	dial.	not dial.	8	dial.	not dial.	9	dial.	not dial.	10	dial.	not dial.	11	dial.	not dial.	12
cells																	
stretch	70		60		90		60		80		50		90		20		30
teating	low		low		high		low		high		high		-		low		low
d.c.	60		30		10		10		-		80		40		-		-
symp. good cells	good cells		good cells		good cells		good cells		good cells		good cells		good cells		fat + 6		fat + 6
Chinese H ₂ cells																	
stretch	100		100		100		90		30				95		50		10
symp. good cells	good cells		good cells		good cells		fat + 8		shrunk				good cells		fat + 8		fat shrunk
rich cells																	
symp. good cells	good cells		good cells		shrunk		good cells		shrunk		good cells		good cells				shrunk



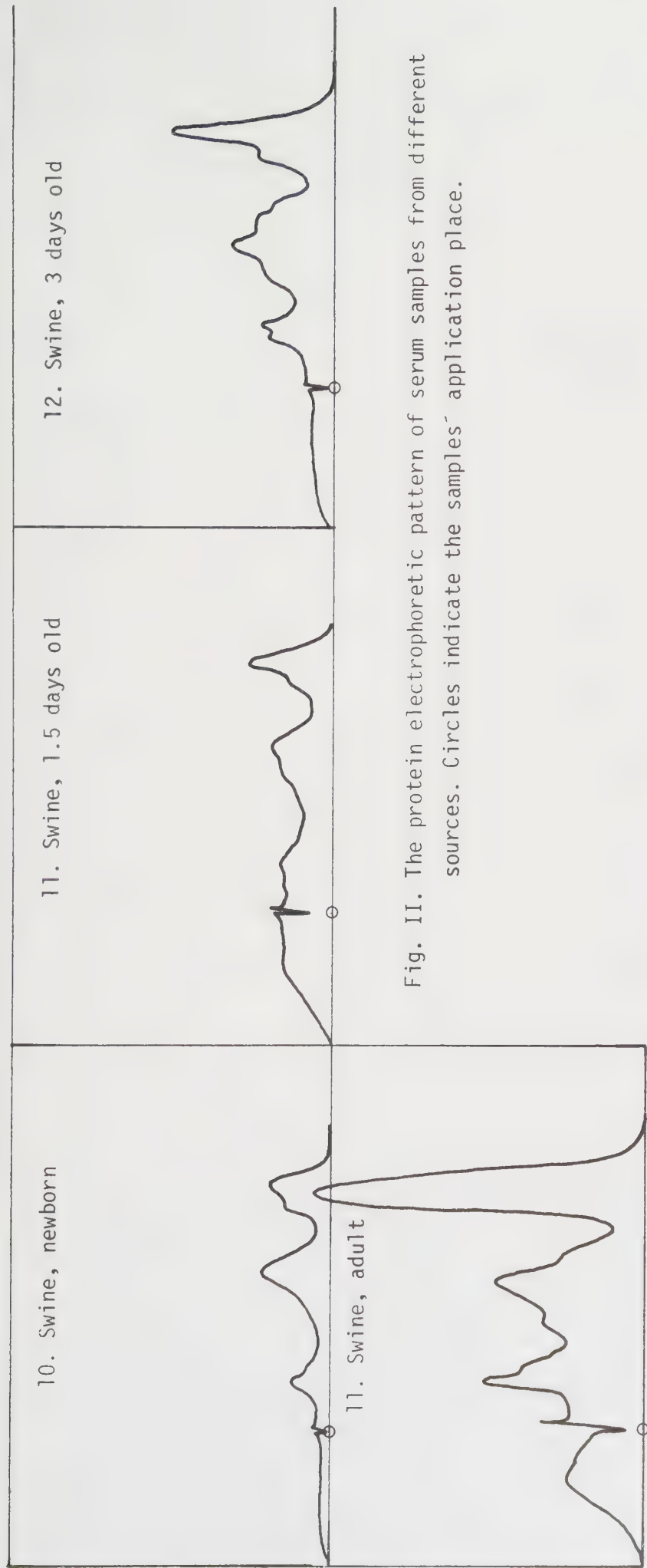


Fig. II. The protein electrophoretic pattern of serum samples from different sources. Circles indicate the samples' application place.

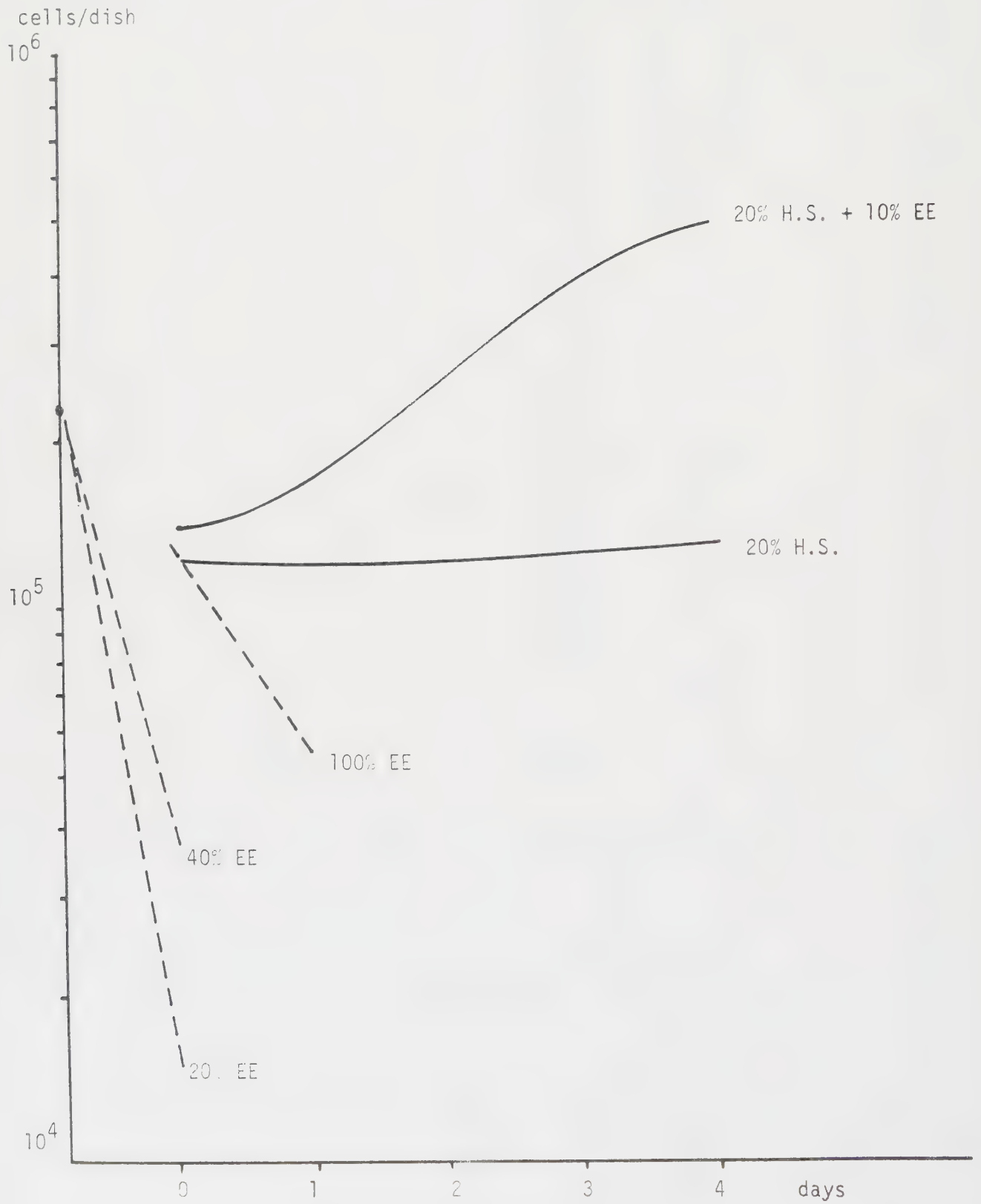


Fig. III. Survival and growth of 12 day embryonic heart cells in different biological fluids.

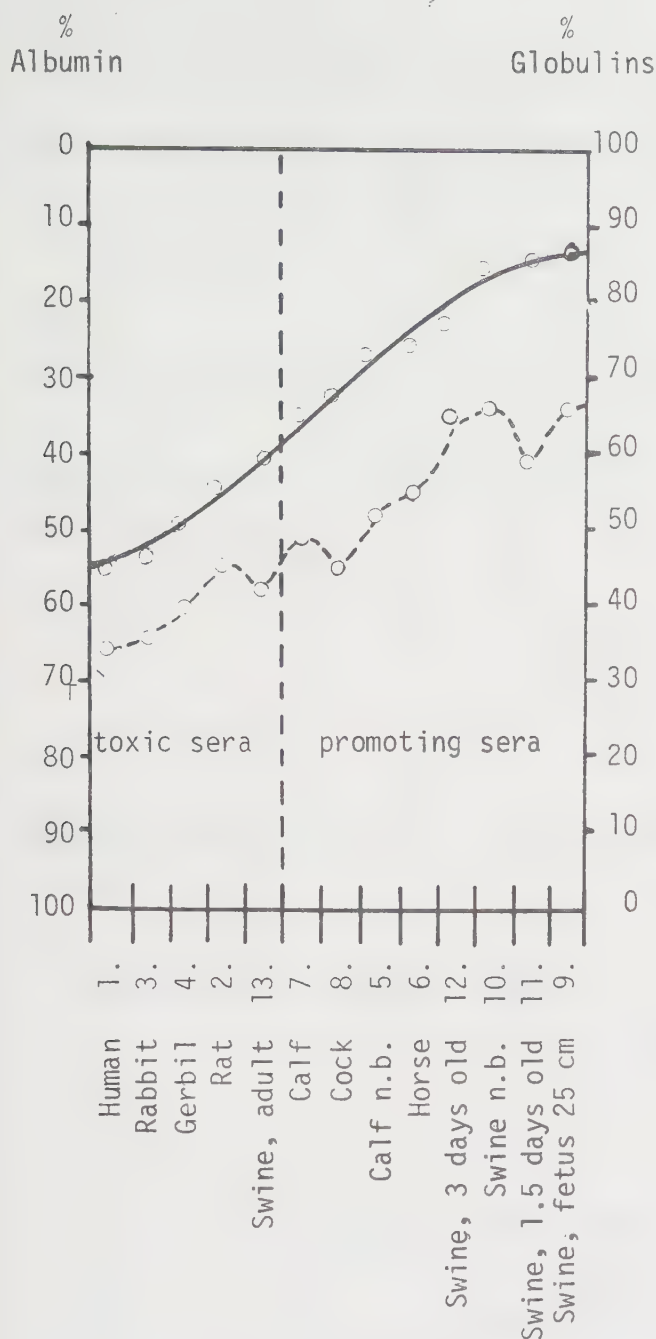


Fig. IV. The upper curve $\circ-\circ-\circ$ indicates the relative levels of albumin and total globulins of different sera. The relative levels of $\alpha+\beta$ globulins are shown in the lower curve $\circ-----\circ$. The space between the two curves indicates the relative contribution of the γ -globulins.

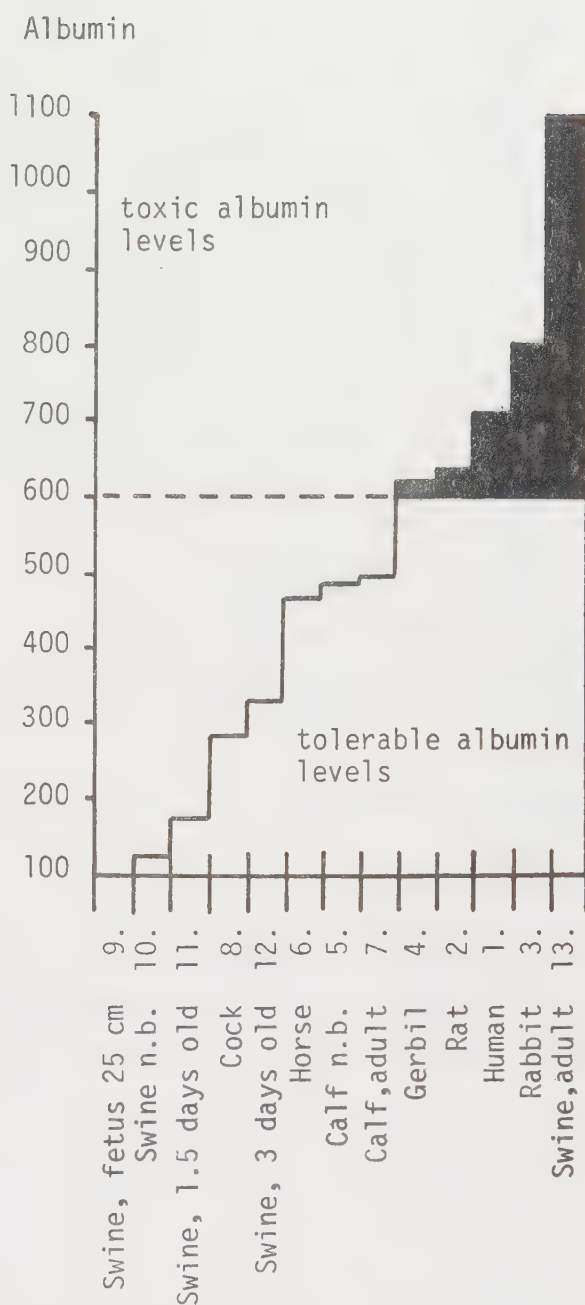


Fig. V. Comparative levels of albumin in different sera; the lowest (serum No. 9) is assumed as 100. Values 6 times and greater than the lowest render the serum toxic.

cells/dish

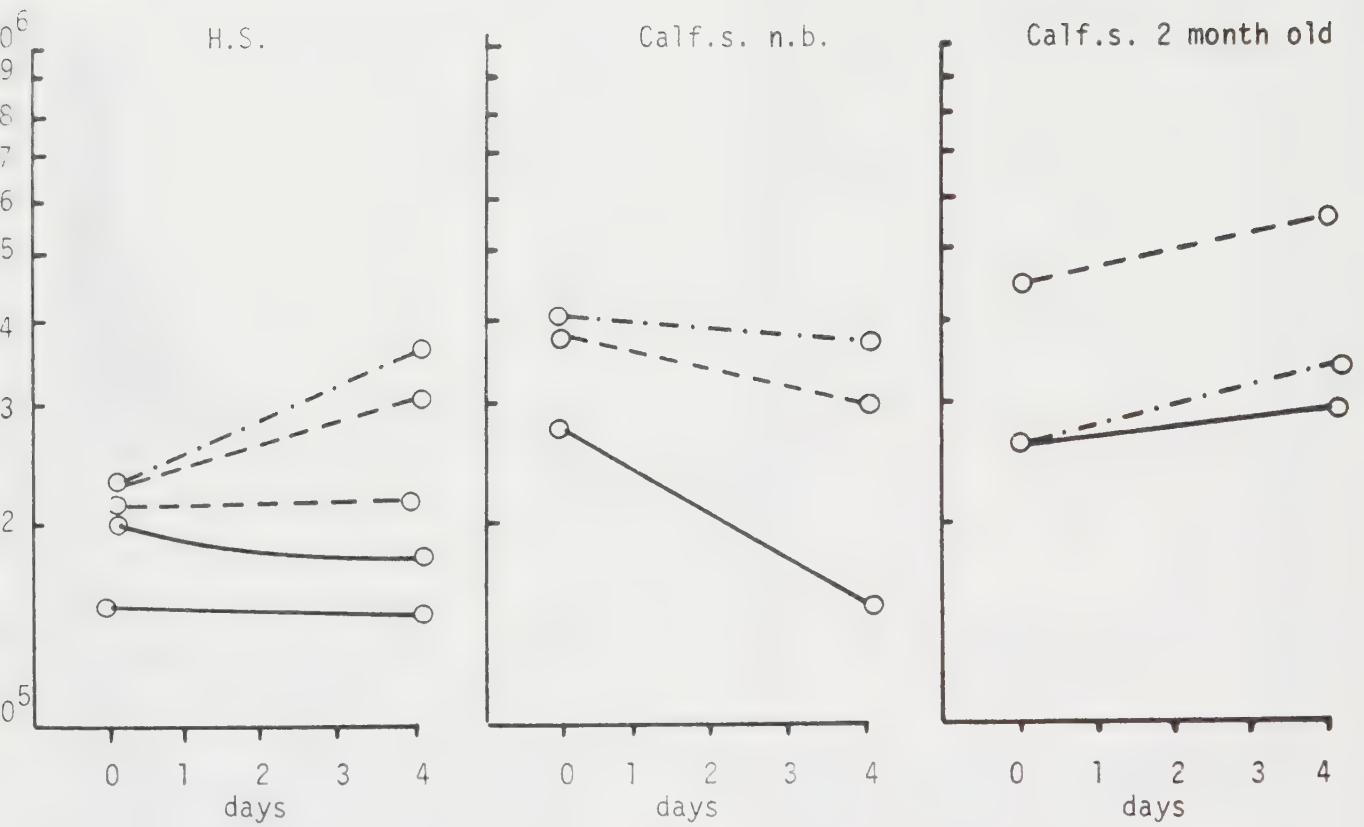


Fig. VI. Growth of 12 day embryonic chick heart cells in normal (not denatured) serum —○—, in denatured serum 56°C for 30 min - - -○-, and in denatured serum 64°C for 12 min - · - · -○-.

cells/dish

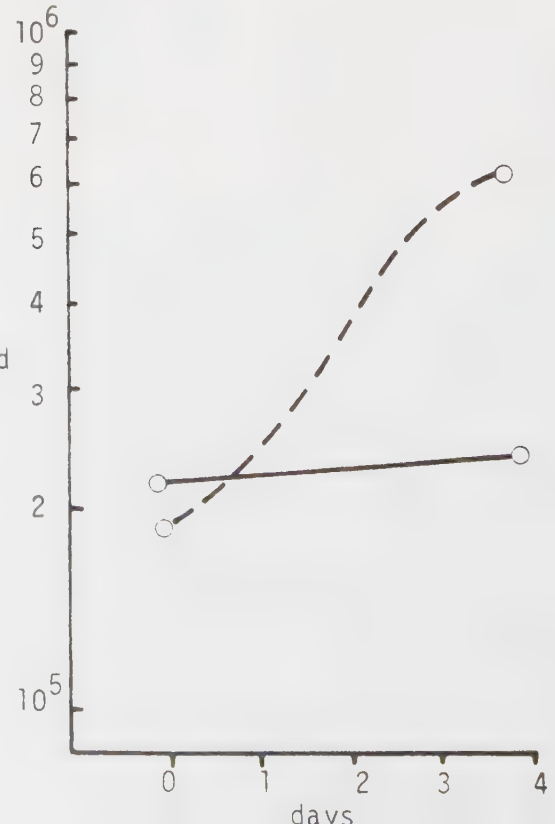


Fig. VII. Growth of 12 day embryonic chick heart cells in 80% Tyrode's BSS supplemented with 20% dialyzed calf serum —○— and in 20% dialyzed calf serum supplemented with 80% synthetic medium M199 - - -○-.

Fig. VIII a.

The protein electrophoretic pattern of the native H.S. ———, and H.S. denatured in 56°C for 30 min - - - -. Circles indicate the samples' application place.



Fig. VIII b.

The protein electrophoretic pattern of the native H.S. ———, and H.S. denatured in 64°C for 12 min - - - -.

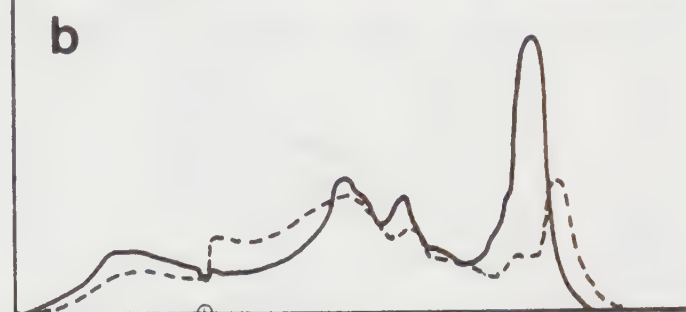


Fig. VIII c.

The protein electrophoretic pattern of the native H.S. ———, and H.S. denatured in 80°C for 2,5 min - - - -.



Fig. IX.

The protein electrophoretic pattern of the 50% D (56°C, 30 min) H.S. ——— and a mixture of 50% D H.S. + 20% EE - - - -. The latter's protein level found to be lower, as the addition of 20% EE diluted the serum in a corresponding proportion.

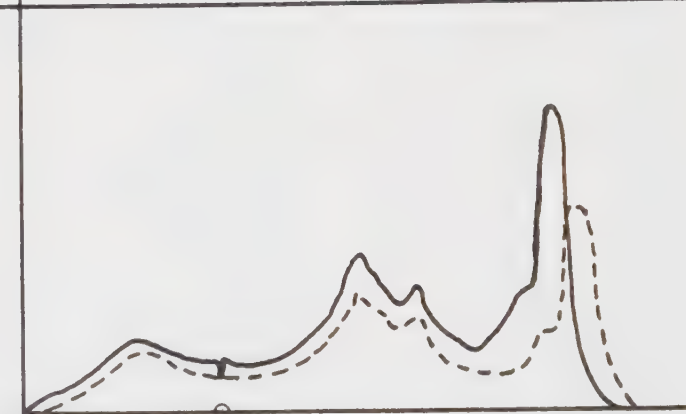


Fig. X.

The protein electrophoretic pattern of native EE ———, DEE in 56°C for 30 min - - - -, DEE in 64°C for 30 min - · - · - ·, and DEE in 80°C for 30 min - x - x - x -. The actual amount of HMW fraction of EE compared with the serum (see above figures) is much lower than is shown in this figure, as the serum was diluted 50% and 10 µl of EE was applied for electrophoresis. See also Material and methods.



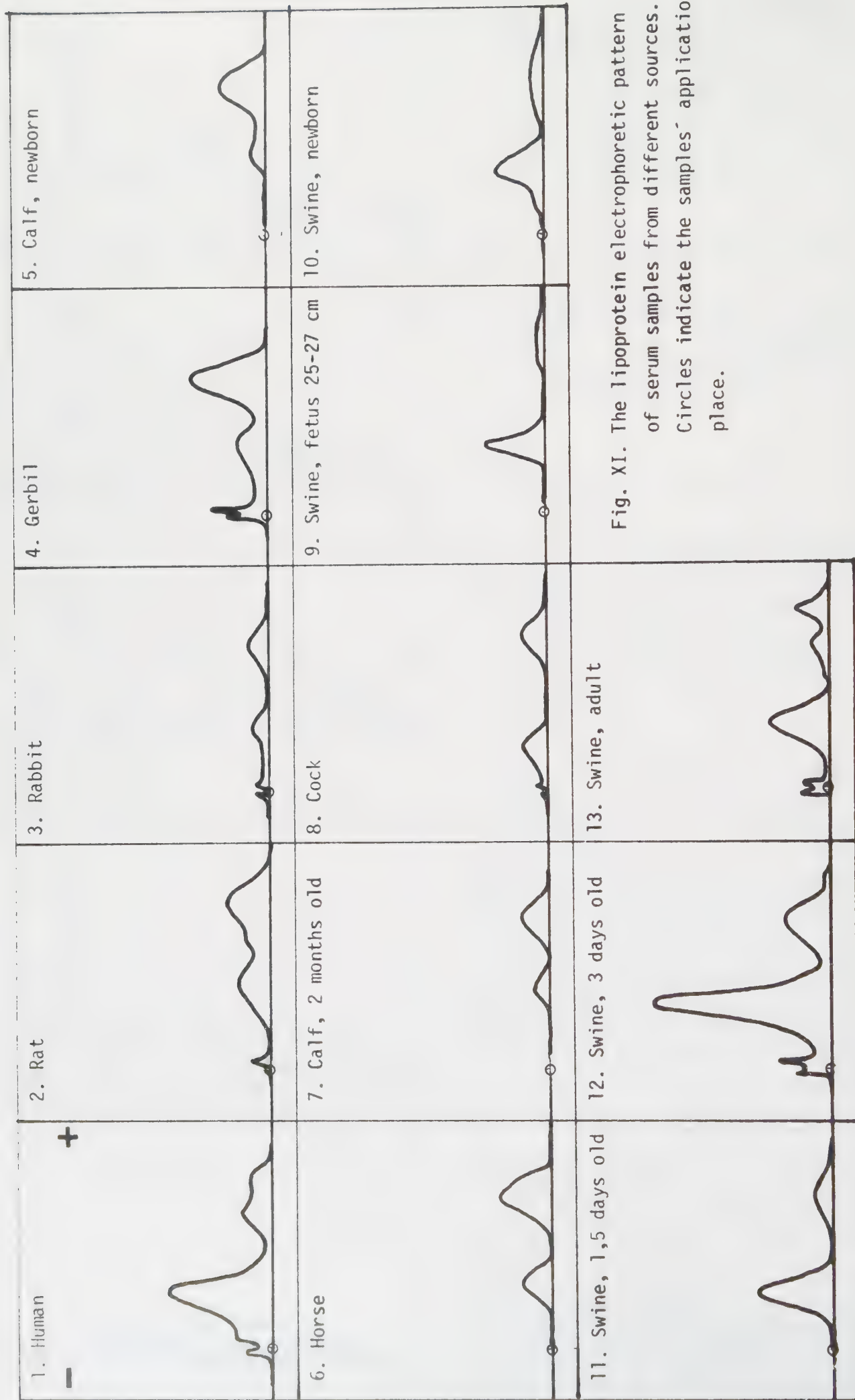


Fig. XI. The lipoprotein electrophoretic pattern of serum samples from different sources. Circles indicate the samples' application place.

No	Serum	1 Total protein (T.P) ★	2 rela- tive levels of T.P. ★★	3 Total albumin	4 rela- tive levels of Alb.	5 % Alb. in the same serum	6 Total γ-glo- bulins	7 rela- tive levels of γ- glob.	8 % γ in the same serum	9 Total α+β	10 rela- tive levels of α+β glob.	11 % α+β in the same serum	12 Total α+β+γ	13 rela- tive levels of α+β+γ glob.	14 % α+β+γ in the same serum	15 A/G ratio
1	Human	673	180	342	713	54	75	114	12	256	136	34	331	119	46	1.17
2	Rat	700	198	302	629	44	77	117	11	321	171	45	398	143	56	0.79
3	Rabbit	722	205	388	808	54	71	108	10	263	140	36	334	120	46	1.17
4	Gerbil	616	175	300	625	49	70	106	11	246	131	40	316	114	51	0.96
5	Calf, n.b.	853	242	230	479	27	169	256	20	454	241	53	623	224	73	0.37
6	Horse	831	235	220	458	26	160	242	19	451	240	55	611	220	74	0.35
7	Calf, 2 month old	646	183	235	490	36	95	144	15	316	168	49	411	148	64	0.56
8	Cock	414	117	136	283	33	90	136	22	188	100	45	278	100	67	0.49
9	Swine, fetus 25 cm	353	100	48	100	14	66	100	19	239	127	67	305	110	86	0.16
10	Swine, n.b.	387	110	58	121	15	71	108	18	258	137	67	329	118	85	0.18
11	Swine, 1.5 days old	588	167	84	175	14	166	252	28	338	180	58	504	181	86	0.16
12	Swine, 3 days old	707	200	158	329	22	92	139	13	457	243	65	549	197	78	0.28
13	Swine, adult	1265	371	530	1100	42	210	318	16	525	279	42	735	264	58	0.72

Table 2

★ Total values (column 1,3,6,9,12) indicate the numbers obtained by reading the electrophoretic curves in the Chromscan.

★★ The relative levels of different components in the 13 sera (column 2,4,7,10,13) were calculated by assuming the lowest value in the serie as 100 _____.

	1	2	3	4	5	6	7	8
	N.S.	D.S. 56°C 30 min	D.S. 64°C 12 min	D.S. 80°C 2,5 min	N.S.	D.S. 56°C 30 min	D.S. 64°C 12 min	D.S. 80°C 2,5 min
Albumin	100	109	45	29	26	25	14	14
α_1 -region	100	133	72	49	7	9	6	7
α_2 - "	100	100	91	29	17	15	17	} 80
β - "	100	116	127	72	31	33	45	
γ - "	100	100	76	38	19	18	17	
Total protein	100	110	86	47	100	100	100	100

Table 3

Quantitative changes occurring in the serum by different heat denaturation treatments.

The left part in Table 3 shows the changes in absolute values when different fractions of N.H.S were assumed as 100. The right part in Table 3 indicates the changes occurring in relative values of different fractions when total protein in the same serum is assumed as 100.

CHAPTER 2

INHERENT GROWTH CAPACITY OF CHICKEN HEART CELLS FROM DIFFERENT
EMBRYONIC AGES^{x)} IN CULTURE

x) The results of the present chapter have been partly published by
Agrell, I. and Gabbay, M. in Experimental Cell Research 82, 131-136, 1973.

MATERIAL AND METHODS (for chapter 2 and 3)

- 1) Preparation of embryonic heart cells. White Leghorn hen eggs were incubated in a humidified incubator ($37-38^{\circ}\text{C}$) for 5, 12, and 18 days. The embryos were removed aseptically and put in Tyrode's BSS. The hearts were taken out and placed in another Petri dish containing Tyrode's BSS, and the ventricle + atriae were cut into c. 0.5 mm^3 pieces with a sharp knife in the presence of some drops of Tyrode's BSS. Additional drops of Tyrode's BSS were added to these pieces with the Petri dish tilted; thus most of the red blood cells were washed away. The tissues were then placed in a 25 ml Erlenmeyer flask containing 5 ml of 0.05% trypsin (crystalline trypsin Novo Industry A/S) in Ca^{2+} and Mg^{2+} free Tyrode's BSS and incubated at 37°C on a shaking device (about 200 shakes/min).

The duration of trypsinization varied according to the embryonic age. The disintegration of the entire tissue took 1.5-2 h for 18-day hearts and 1-1.5 h for 12-day hearts; for 5-day hearts, 30-40 min were sufficient.

Finally, in order to make a homogeneous single cell suspension, the tissues were pipetted up gently 10-20 times by means of a 2 ml Pasteur pipette (1 mm diameter at the opening) and the sample was counted in a Bürker chamber. The cell suspensions were diluted into at least 5 different initial concentrations with prewarmed Ca^{2+} and Mg^{2+} free Tyrode's BSS, and 2 ml of each were explanted in duplicates into plastic Petri dishes (Falcon 35 x 10 mm) and left undisturbed on a flat surface for 15-20 min. During this period, almost all the cells settled to the plastic surface (4). The trypsin solution was then removed and replaced with 2 ml of prewarmed basal medium (30% H.S. denatured at 56°C for 30 min + 70% Tyrode's BSS) and transferred to a humidified incubator at 37°C in an atmosphere of 5-6% CO_2 in air for the next 20-24 h. During this period, between 50 and 70% of the cells definitely attached and flattened out. (With inoculum sizes of about $500-600\text{ cells/mm}^2$, most of the cells attached). The basal medium was then sucked off and replaced by 2 ml of prewarmed growth medium (20% DH.S. + 10% EE + 70% Tyrode's BSS). During the subsequent incubation, the proliferation, differentiation, and spontaneous activity were evaluated. The proliferation was followed by daily counting of the cell nuclei in the same series of fields, 1 mm^2 each on the Petri dish in

the phase contrast microscope, 8-10 fields/dish, until the culture reached a stationary phase without any change of the medium. The percentage of differentiated cells was counted after the cells were fixed and stained while the stationary phase of growth was being reached (Chapter 2), or the cells were fixed and stained in different stages of culture period (Chapter 3). The percentage of d.c. was obtained as follows: The stained cultures were observed throughout under high magnification (1000 X) immersion oil objective, and any cell that showed any degree of striation was counted as d.c. At least 30-40 fields, representing 600 and more cells/dish, were evaluated. The counting was continued until at least 5 approximately similar percentages were obtained.

All the culture procedures were carried out under highly sterile conditions; thus antibiotics were unnecessary. All solutions were filtered in Millipore filters 0.45 μ m pore diameter. The material in Chapter 2 represents at least 8 series of experiments for each age. About 3/4 of the experiments were performed according to the above procedure. However, in order to minimize the external influences on the direct relation between the embryonic age and the proliferation of the cells in vitro, the remaining 1/4 of the experiments were performed in standardized conditions. Thus, the differential effect of trypsin dissociation on the cells was equalized by placing in trypsin solution approximately the same quantity of tissue from 5-, 12-, and 18-day embryonic hearts and disintegrating them for an identical length of time (60 min). The 9-day EE was either heat inactivated at 56°C for 30 min, or the 5-, 12-, and 18-day heart cells were cultivated in extracts prepared from 5-, 9-, and 14-day embryos.

All the Chapter 3 materials were treated in standard conditions and represent at least 8 series of experiments for each age.

- 2) Preparation of EE. The EE was prepared as described by J. Paul (75). Embryos were aseptically removed from eggs incubated for 9 days, rinsed three times in Tyrode's BSS, and put into a syringe. They were then expressed into a centrifuge tube. An equal volume of Tyrode's BSS was added to the pulp, which was stirred with a sterile glass rod and left for 30 min at room temperature and then centrifuged for 30 min at 3000 rev/min. The supernatant (EE₅₀) was distributed in 10 ml test tubes and deep-frozen (-20°C). Before use, the EE was again centrifuged for 20 min at 3000 rev/min.

- 3) Method of staining. The Heidenhain Iron Hematoxylin method, as described by Gurr (30), was used. The culture medium was sucked off and replaced by prewarmed Tyrode's BSS for 1-2 min. This was repeated three times; the cells were then fixed in a mixture of absolute alcohol, acetone, and acetic acid, 3:1:2, for 10-15 min. Rinsing through 96%, 70%, 50%, 30% alcohol and distilled water followed. A solution of 3% iron alum was added to the fixed cells for 3 h. It was then replaced after the dish had been rinsed three times in distilled water by a solution of 0.5% Hematoxylin for 1-2 h. The stain was differentiated with 0.01% iron alum solution and the preparation was washed for 20 min in tap water. Dehydration followed in the alcohol series (30%, 50%, 70%, 96%, and absolute alcohol). The cultures were finally put under a cover glass, non-drying immersion oil^{x)} being used as the mounting medium.

RESULTS

Chicken myoblasts of different embryonic age were cultivated in growth medium (20% DH.S. + 10% EE) without any change of nutrition medium, and the growth was followed to the stationary phase.

In these closed systems, the terminal cell density was found to depend on the initial amount of cells in the following three ways (Fig. 1):

1. At high initial cell concentrations, growth decreased or ceased, presumably owing to the crowding within the closed cultivation system (71).
2. At low initial cell concentrations, growth also decreased or ceased, probably because of low-conditioned medium (87).
3. Within an intermediate range of initial cell concentration, where irrespective of the embryonic age of the myoblasts the highest value was about ten times the lowest, the growth rate was fairly constant for each age; the higher the initial cell concentration, the higher the final amount of cells. The most remarkable result obtained was that these types of dependency of the amount of growth on the initial cell concentration quantitatively differed for cultivated myoblasts of different embryonic age. Thus myoblasts

x) Cargille Laboratories, non-drying immersion oil type B.

of each age displayed a different degree of interdependence and different growth properties.

Moreover, cells from each age showed a different degree of interrelation for growth by requiring different inoculum cell densities, i.e. the initial cell concentration necessary for growth was somewhat higher for the younger ages; for instance, whereas initial cell densities approximately below 40 cells/mm² did not allow growth of 5-day cells (Fig. 1), the 18-day cells could start to proliferate in a lesser initial cell concentration that amounted to about 20 cells/mm². In this respect, cells of 12-day embryos more resembled the 18-day than the 5-day, the lower limit for growth being between 20 and 40 cells/mm². This means that, for survival and growth, cells from younger embryos depend more on each other and on conditioning effect than cells from higher ages (Fig. 1). Concerning the upper limit of initial cell concentration and the growth of cells from different ages, the growth of 5-day cells diminished already when the initial cell concentration approached around 800-900 cells/mm² (Fig. 1) (by gradual decrease in rate and shortening of the duration of the growth). But cells of higher ages in the same initial cell concentration still continued to grow, i.e. the diminution of growth appeared in higher cell densities, suggesting that, in respect of the crowding effect, the cells from 5-day embryos were affected earlier than cells from higher ages. Thus the limit of initial cell concentration necessary for growth was somewhat more restricted for the younger ages (4-5 day) than for the older ages (18-19 day). It is especially remarkable that, within the intermediate range of cell concentrations that allows optimum growth, the final amount of growth for older is much greater than for younger embryonic stages, i.e. the growth capacity of cells in culture increases with the increase in embryonic age. For instance, although the final amount of growth of 5-day cells never exceeded 3 times the initial amount (the common values for F/I being around twice) (Fig. 2), this increase for the cells of 18-day embryos was the lowest value of F/I, and the highest final growth could, in some cases -- i.e. in low inoculum sizes -- reach values of 10 times the initial amount. The cells of intermediate age, i.e. the 12-day, showed also intermediate growth capacity. Thus, the first part of the curves in Fig. 2, which can reasonably be described as straight, suggests that the final amount of cells will be about twice the initial amount for 5-day embryos, about

4 times the initial amount for 12-day embryos and about 8 times the initial amount for 18-day embryos. Additional observations for growth capacities of other embryonic ages revealed that the growth capacity of the cells from embryos aged more than 18 days, for instance, 19 and 20 days, is higher than that of cells from 18-day embryos and for cells from embryos aged less than 5 days is even less than that of cells from 5-day embryos. On the other hand, cells cultivated from 7-day embryos already showed a growth pattern more like those from the 12-day than from the 5-day embryos. However, cultivation of cells from additional intermediate embryonic ages, i.e. 9, 14, 15 and 17, did not reveal a gradual, but an intermediate, growth capacity, resembling the growth of 7- and 12-day embryonic cells. Thus, no gradual increase in growth capacity was observed between 5- and 18-day cells, but the difference in growth capacity between 4-5-day and 18-19-day embryos was highly significant. The other ages in between, i.e. from 7- to 17-day showed a similar and intermediate growth capacity corresponding to that of 12-day embryo cells. The different growth capacity that increased with the developmental stage is determined by two factors, as Fig. 1 shows.

1. A different duration of growth, i.e. whereas growth of 5-day cells, continued for 3 days until the stationary phase was reached, for 12-day cells, a growth period of 4 days was necessary to reach the stationary phase, and the 18-day cells needed 4-6 days to reach the stationary phase. Depending on inoculum size, the growth period varied (in relatively low inoculum sizes, growth continued for a longer time; in relatively high inoculum sizes, the growth period was shorter).
2. A different growth rate¹⁾ in exponential phase; thus the average growth rate for 5-day cells was 1.35, for 12-day cells 1.58, and for 18-day cells 1.77 per day.

This sequence of growth rate in vitro is opposite that in vivo. From Ramanoff (85) Table 6, wet weight, one can calculate the growth rate of the chicken embryo heart during the embryonic development. This growth rate is for 5-6-day embryos 1.92, for 12-13-day embryos 1.38, and for 18-19-day embryos 1.21.

1) The calculation of growth rate was made according to Ramanoff (85).

$\frac{W_2 - W_1}{W_1} \times 100$, i.e. percentage of increase in amount per unit of time (day). W_1 and W_2 indicate cell count on successive days.

In a few cases, the growth period was identical for all the different ages observed, 5-, 12-, and 18-day cells; despite this, the growth rate was different, being higher for higher ages and lower for lower ages. On the other hand, in some other cases, the total growth was similar in cells of different ages, i.e. the growth was allowed to reach a certain level in all ages irrespective of their inherent capacity. However, this restriction of total growth capacity was found to be correlated with the quality of the nutritional medium. Thus the function of the nutritional medium was found to be crucial concerning its ability to allow maximum growth capacity to be expressed. Fig. 4 and 5 in Chapter 3 show this correlation. As Fig. 4 in Chapter 3 shows, growth is proportional to the concentration of EE, i.e. in low concentrations, the 18-day cells with a high inherent growth capacity grow similar to 5-day cells. But 5-day cells do not grow beyond a certain level, even if placed in a high concentration of EE, because of their limited inherent growth capacity. Moreover, the 18-day cells in denatured EE (Fig. 5, Chapter 3) might not show their maximum growth capacity.

An additional phenomenon was the variations in the lag period of growth curves of cells under identical condition. The lag period was shown to depend on at least two major factors: 1. The inoculum size, i.e. an initially lower cell concentration provoked a longer lag period (Fig. 1); 2. The embryonic age, i.e. whereas a latent period of 1 to 2 days was almost always present in cultures of cells of higher ages (e.g., 18- and 12-day cells) (Fig. I), this phenomenon was rarely noted in cultures of 5-day cells. An additional factor that affected the duration of lag period was the quality of the medium, i.e. in a medium rich in essential metabolites for growth, such as EE (Fig. 5, Chapter 3), the lag period was much shorter.

DISCUSSION AND CONCLUSIONS

The principal result of the foregoing experiments is the established observation that, for chick myoblasts of different embryonic ages cultivated in vitro, the amount of growth depends on the initial cell density, which is critical, and in turn determines the rate and duration of the growth. This quantitative growth pattern differed in myoblasts from embryos of different ages. According to Earle et al. (21), mouse cells, L strain in suspension cultures, grow independent of the size of the inoculum; thus the final amount of cells will be constant. This means: "cultures that survived and were planted with graded sizes of inoculum, all reached approximately the same concentration of nuclei by the third week of growth". The same independence of the growth, concerning the size of the inoculum, is observed for ascites cells grown peritoneally in mouse (50). However, these mentioned examples of cultures are to a certain extent "open", because, for the L strain cells, the medium was regularly renewed (3 times a week), and the ascites fluid was continually drained by the host, the mouse. On the other hand, the myoblast cultures in the present study represent closed systems, because the medium was not renewed during the whole of the studied growth period. The effect of initial cell density in a closed system on the rate of growth is well shown in growth behaviour of Ehrlich ascites tumour cells in vitro (Fig. 3). Even for these cells, which have a rather unlimited growth potential, the final growth is limited and depends on the initial cell concentration when the cells are placed in a closed system. Earle et al. (21) found that the lower limit of cell density that allowed survival and proliferation of L cells is determined by the volume of culture medium that the cells are bathed in. They state: "the volume of culture fluid to be utilized and altered by each cell may be a critical limiting factor in determining the minimum number of cells that will survive and proliferate" (in suspension cultures). They found this volume to be 0.04 mm^3 per cell, corresponding to the optimum volume needed for cloning a single L-cell in a capillary tube.

The limiting low density growth factor was reported by Rein and Rubin (82). All growth potential of a chick embryo cell population was lost within 24 hours if the cells were plated at a low concentration. Rein and Rubin (83) also stated that the density of cells per unit area of the substratum rather than the total number of cells per culture vessel

or per unit volume of medium determines the growth of chick embryo cells. Rubin (87) found this minimum cell density to be 2×10^5 cells/100 mm dish, i.e. about 25 cells/mm² of the substratum, and Todaro et al. (96) found it to be 3×10^4 /50 mm plate (18 cells/mm²). The size of inoculum also affected the rate of proliferation of L-cells (21), i.e. a reduction in the rate of proliferation occurred as the inoculum size approached minimum or maximum levels. The highest rate of proliferation was found to take place in an intermediate inoculum size. Rubin (87) reported similar results. He found that growth rate decreased with decrease in initial cell concentration. Rein and Rubin (83) further stated that the growth rate of secondary chick embryo cells is positively correlated with their cell concentration over a wide range. Stoker (94) concluded: "in general growth rate is directly related to cell concentration at low density, but inversely related at high density, so that growth slows or ceases in crowded cultures of either attached or suspended cells"; moreover, he hypothesized the possible action of crowding on lack of growth, being due either to the depletion of essential nutrients or to the action of one or more inhibitory factors. (Actually he presumed the latter phenomenon to be the real factor in reducing growth in crowded cultures, as the evidence opposes the former). Kohn (52) observed that, despite frequent replenishment of the medium, the growth ceases in confluent cultures.

On the other hand, Rubin (87) showed that the presence of conditioned medium can enhance the growth of a small number of cells, the rate of growth depending on the concentration of the conditioned medium. The conditioning factor seems to be composed of very large molecules or large aggregates of smaller molecules acting in the immediate vicinity of the cell (87, 83).

The results of the present study agree with the mentioned conclusions that the limits, the rate, and the amount of growth depend on initial cell densities. The present study also demonstrated that this growth behaviour differed significantly in cells from different embryonic ages when cultivated in vitro. In the optimum range of initial cell concentration for growth within one and the same cultivation dish, the initial number of cells within the various observation fields counted varied about fivefold. Despite this varied initial density, all fields showed almost the same pattern of growth. This can be seen in Fig. 2 where the points do not

represent mean values, but the actual counts of the cell number in each observation field. Any interaction between the cells seems to be ruled out. Thus the cultivated myoblasts demonstrated no mutual feeding effect (71, 94), no mutual nuclear activation (11), and no topo-inhibition (81). Only a specific inherent capacity for growth seems to act differently for varied embryonic ages. Thus the growth capacity of the cells in culture increases with increase in embryonic age. This inherent capacity is not independent of the medium. The medium must basically be able to support maximum growth. For instance, without EE no myoblast proliferation will occur (Fig. 2, Chapter 3) only an increase in size of the single cells (4). The inherent growth capacity was seen to increase with increasing embryonic age. This result differs from those obtained by other authors who studied varying embryonic tissues, including heart. For instance, Carrel (15), comparing growth of heart fragments from 10- and 17-day embryos, found a marked difference in the inherent activities of the two tissues, i.e. the growth of 17-day tissue was 30% less extensive than that of the 10-day tissue, which implies a decrease in inherent activity with increase in age. Chaytor (18) reported similar mitotic indices after a short period of culture of chicken heart explants from 7- and 14-day embryos. Medawar (63) states that chicken's heart-growth energy declines exponentially with age. Hoffman et al. (42), cultivating chicken heart fragments from 7- and 21-day embryos and 1-year old chicken, found the same growth capacity for adult fragments as for those from the embryos. In one case, the results of the present study were to some extent confirmed elsewhere. Thus Parker (73), who used explants of chicken embryo skeletal muscle, found that the rate of multiplication of a cell strain derived from 21-day old embryos greatly exceeded that manifested by a cell strain from a younger (10-day) embryo. None the less, both cell strains showed much the same morphological appearance after cultivation in serum. On the other hand, Parker, in another experimental series (74), found that cell strains derived from developing heart behaved differently from those obtained from skeletal muscle, i.e. heart fibroblasts from younger embryos (10-day) proved to be more active. Thus the effect of age on cell strains from developing heart was the reverse of that found on cell strains from skeletal muscle. Furthermore, Parker stated (74) that the ability to multiply depends on inherent capacity of the cells themselves, and this inherent capacity is permanent. He also maintains that it was impossible to demonstrate a gradual increase

or a gradual decrease in the rate of the proliferation of the isolated cell strains with gradually increasing embryonic age.

The present investigation, besides finding an inherent growth capacity of the disintegrated heart cells, established that the growth capacity increases with increasing age of embryo donor. This is particularly obvious when the two extreme ages, 5-day and 18-day, are compared.

All the above-referred to investigations deal with explants, not with cell cultures. The growth of explants includes, besides cell multiplication, cell migration (18, 21) as an important component. An older explant, for instance, might have a slow migratory rate of cells, but a proliferation rate higher than explants from younger embryos. Different organs can have different migration rates, irrespective of proliferative capacity. Thus explant culture is unreliable for comparing different growth capacities. In this connection, Parker (73) stated that the residual energy (i.e. growth) measured as migration rate, as shown by heart cells, is among the lowest found among the tissues isolated from the chick embryo. Therefore the behaviour of explants "closely-packed cells" (21) and cell cultures are not comparable (100).

An additional phenomenon observed in this study, correlated with the embryological stage of development, is the varying latent or lag period, i.e. cells taken from older embryos needed a longer time in culture to start an active proliferation than those isolated from younger embryos. This finding agrees with those found by Soukupová et al. (92), who studied explant cultures of several organs, including heart, representing various ages: from newborn, adult, and old subjects. They found that the latent period increases during ontogenesis, even when the active embryonic growth of the organism has ceased. Similar results were reported by Hoffman et al. (42), according to whom, explants of chicken hearts from 7- and 21-day embryos and one-year old chicken display progressively lengthened lag periods in vitro before proliferation starts. Examining heart fragments from 4-18-day old chickens, Cohn et al. (1925, cited by Parker in 74) found that the latent period increased with age. Although they did not suggest a possible reason for this behaviour, Parker (74), trying to explain the results obtained by Cohn et al. in explant cultures (see above), thought that there was no direct correlation between specific properties of the cells and the latent periods of cell migration. However, he pointed

out the marked changes that take place in the organs during development; for instance, the gradual increase in the density of connective tissue and its intercellular substances. Therefore older organs necessarily require a longer time to migrate than younger organs, which are less dense and of looser texture.

The present study, which deals with cell cultures as distinct from explant cultures and thus excludes the intercellular factor, shows that a correlation exists between lag period and the developmental age from which the cells are isolated. This suggests a kind of interrelation between the latent period phenomenon and possible changes that occur on the cellular level during development. This theory finds further support from the findings in the present study that the lag period was also dependent on initial cell density.

This phenomenon, on the other hand, was unexpectedly correlated to the time gap required for the cells of different ages to undergo "stretching" or flattening; whereas 95% of 5-day cells stretched already after 24 h, the 18-day cells required 48 h or more to do so. Thus possible changes occurring to the cells in the course of the development, particularly to the cell surface (51), are further emphasized.

When an attempt is made to explain the different inherent growth capacity of cells from embryos of different ages, some preparation artifacts have to be ruled out.

1. As is well known trypsinization or action of other proteolytic enzymes have a promoting effect on cell division (Mazzei et al. (62) and Burger (12)). In the present experiments, the heart tissue from older embryos had to be trypsinized longer than tissue from younger embryos in order to obtain a maximum yield of cells. Could this difference in trypsinization time exert some influence? Repeated experiments where the trypsinization time varied -- prolonged for younger and diminished for older embryos (Fig. 4) -- demonstrated no change whatever in growth. Could the growth possibly be affected by the minimum time for trypsinization which all embryonic heart tissue had to undergo? A mechanical separation of the heart cells in Ca^{2+} and Mg^{2+} free medium only is difficult. Only once was that experiment successful, sufficient and apparently healthy cells being obtained from 12-day embryos. However, their growth capacity was not less than that of the corresponding cell material obtained by trypsinization (Fig. 4).

2. The cells of different ages used in this investigation were cultivated under standard conditions; among other things, standardized serum and EE were used. The specificity of the serum was destroyed by denaturation at 56°C for 30 min. This was also done (in 6 series of experiments) with the EE. This precipitated some protein (Fig. X, Chapter 1), but did not basically affect the growth-promoting action of the extract (Fig. 5, Chapter 3).

3. Extracts from 9-day embryo were usually used. Could this EE possibly affect cells of different embryonic age differently, for instance, promote growth of older embryonic cells, but retard growth of younger ones? Tests made with 5-, 12-, and 18-day myoblasts grown in 5- and 14-day embryonic extract (Fig. 5) exhibited no difference in growth pattern from the corresponding cultures grown in 9-day embryonic extract.

4. It has been argued (20, 78, 79, 80) that embryonic heart cells in culture usually resemble either muscle cells or fibroblasts. In cultures of 4-18-day embryonic heart cells, the muscle-like cells (M-cells) after 24 h comprise 50-60% and the fibroblast-like (F-cells) 40-50% of total population, respectively (DeHaan (20)). Does the proliferation capacity manifested by cultivated heart cells from different embryonic ages possibly result from selection of cells during the cultivation procedure? Can an 18-day cell culture possibly have a higher percentage of F-cells with a rapid rate of proliferation than a 5 day culture with a higher percentage of M-cells and a slower rate of proliferation? As Polinger (80) stated: "overgrowth of one cell type by another and not 'dedifferentiation' is the phenomenon occurring in heart cell cultures". Rinaldini (84), however, suggested that the beating myoblast and the inactive fibroblast in heart culture might be two aspects of the same cell. The present study has shown that the capacity for division in vitro is, remarkably enough, related to the differential stage of the myoblasts in vivo. The more differentiated the myoblasts are, the higher the mitotic capacity in vitro, although the mitotic capacity in vivo is just the opposite. Actually, one might have expected that also in vitro similar conditions would prevail, i.e. the higher the differential stage, the lower the inherent capacity for division (5). Obviously, the cells undergo some de-differentiation through the action of the trypsin separation process; among other things, the cross-striation disappears (Plate 3 and 4, Chapter 3). 4-6-day myoblasts

were still able to re-differentiate but for older cells, it was obvious that the older the stage, the smaller the re-differentiation capacity. Thus, for older myoblasts cultivated in vitro, the general increase in mitotic activity of the cell culture is not inhibited by any re-differentiation; thus their proliferation capacity will be high. The question about cell types in culture derived from the heart and the possible existence of an antagonism between cell proliferation and differentiation are extensively discussed in Chapter 3.

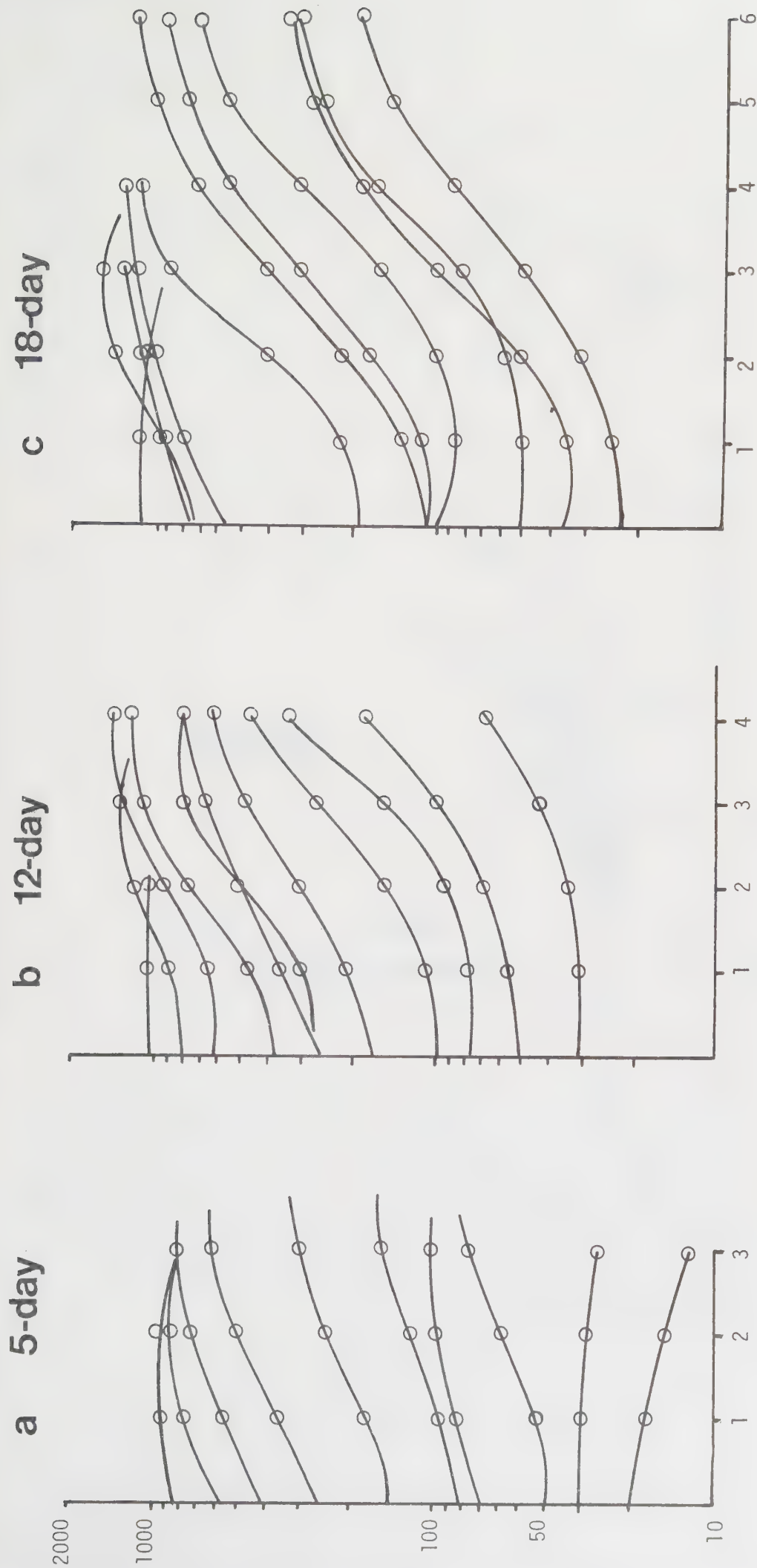


Fig. 1. Abscissa: days, ordinate: cells/observation field (1 mm²). Typical examples of growth curves of disintegrated heart cells from a) 5-, b) 12-, and c) 18-day chick embryos.

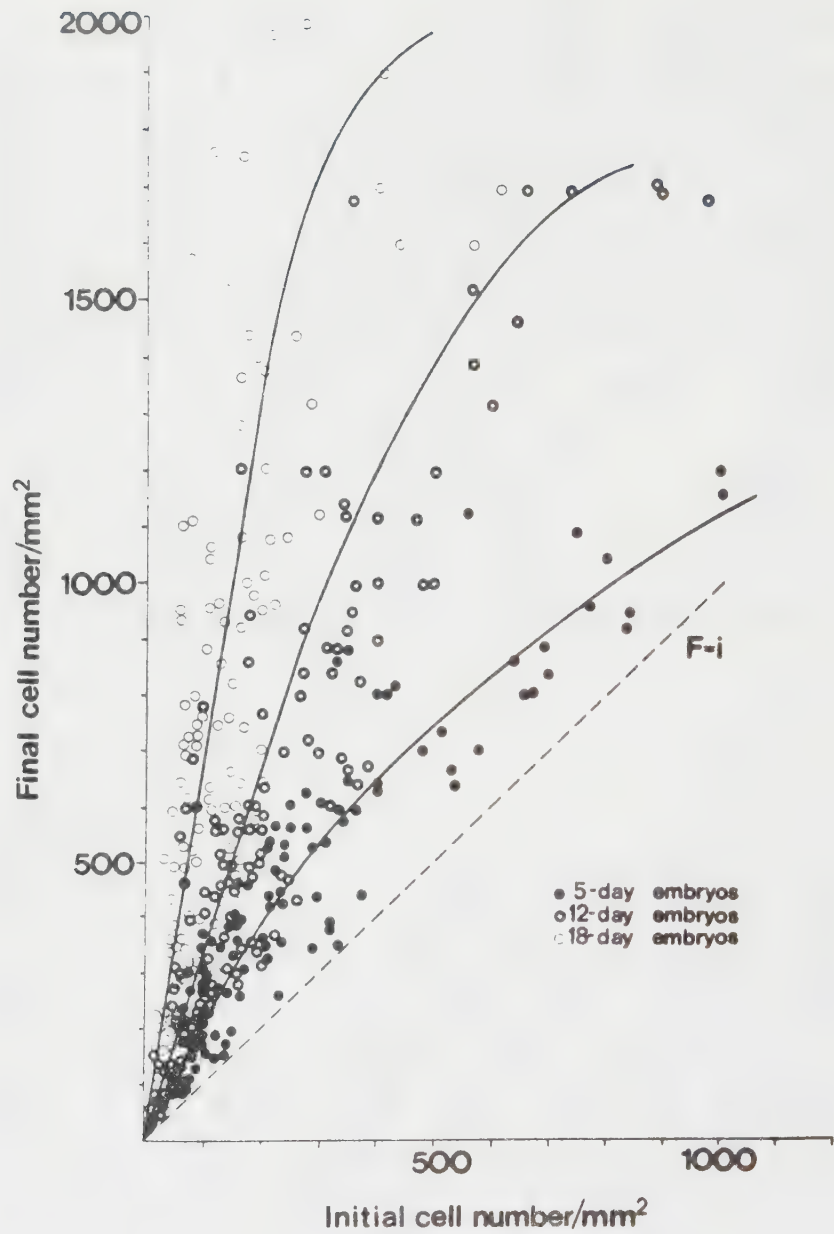


Fig. 2. Abscissa: initial cell concentration/observation field (1 mm^2) (I), ordinate: final cell concentration/field (F). $\bullet$: 5-day, $\odot$: 12-day, $\circ$: 18-day. Each point represent one observation field. The dotted line represents $F = I$, i.e. no growth. (After Agrell and Gabbay, with permission from Academic Press Inc: Expt. Cell Res. 82, 131-136, 1973).

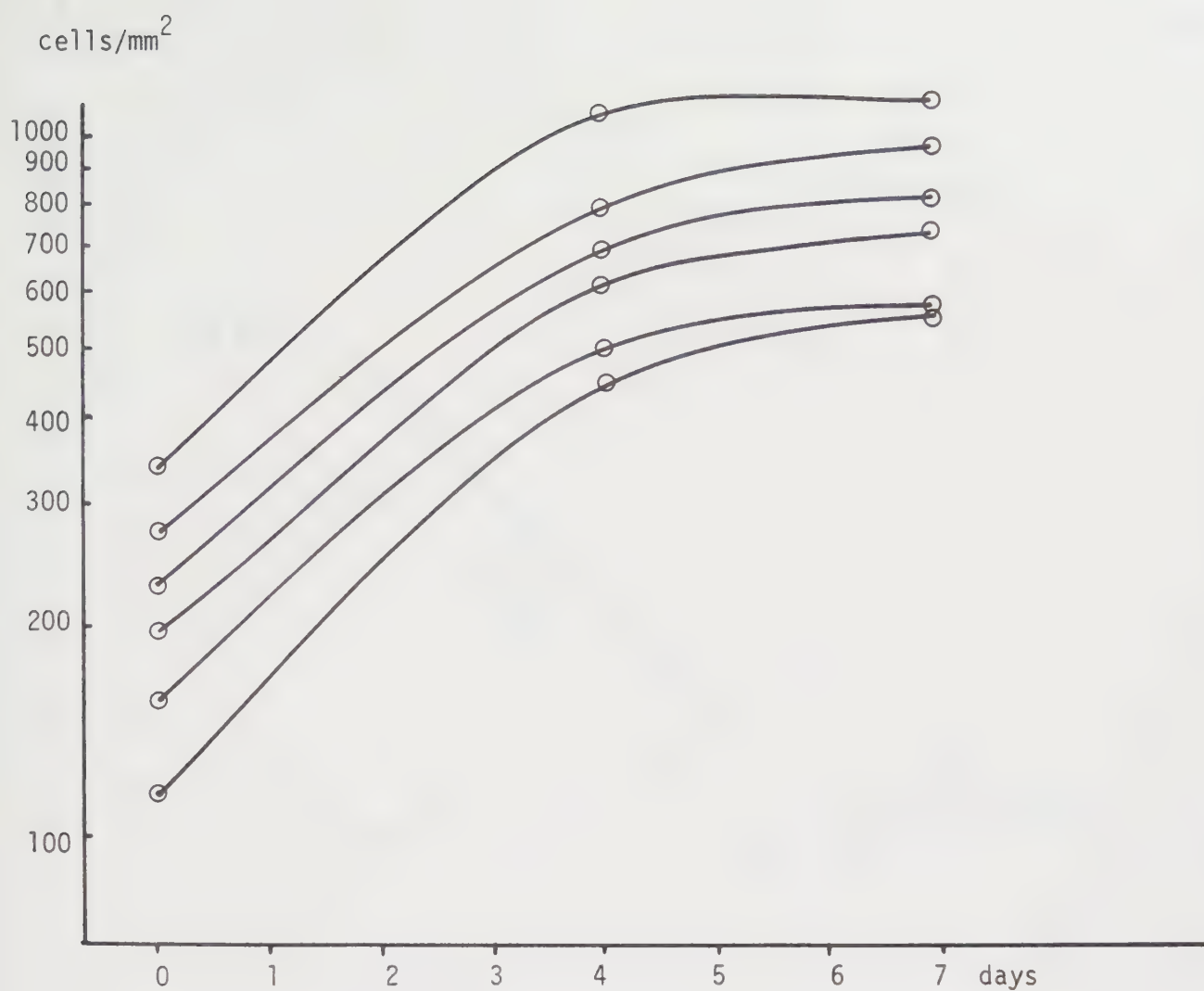


Fig. 3. Growth curves of Ehrlich tumour cells in medium containing 20% newborn calf serum + 80% Eagle (BM) medium. In a closed system, the final cell number is limited and depends on the initial cell number.

cells/dish

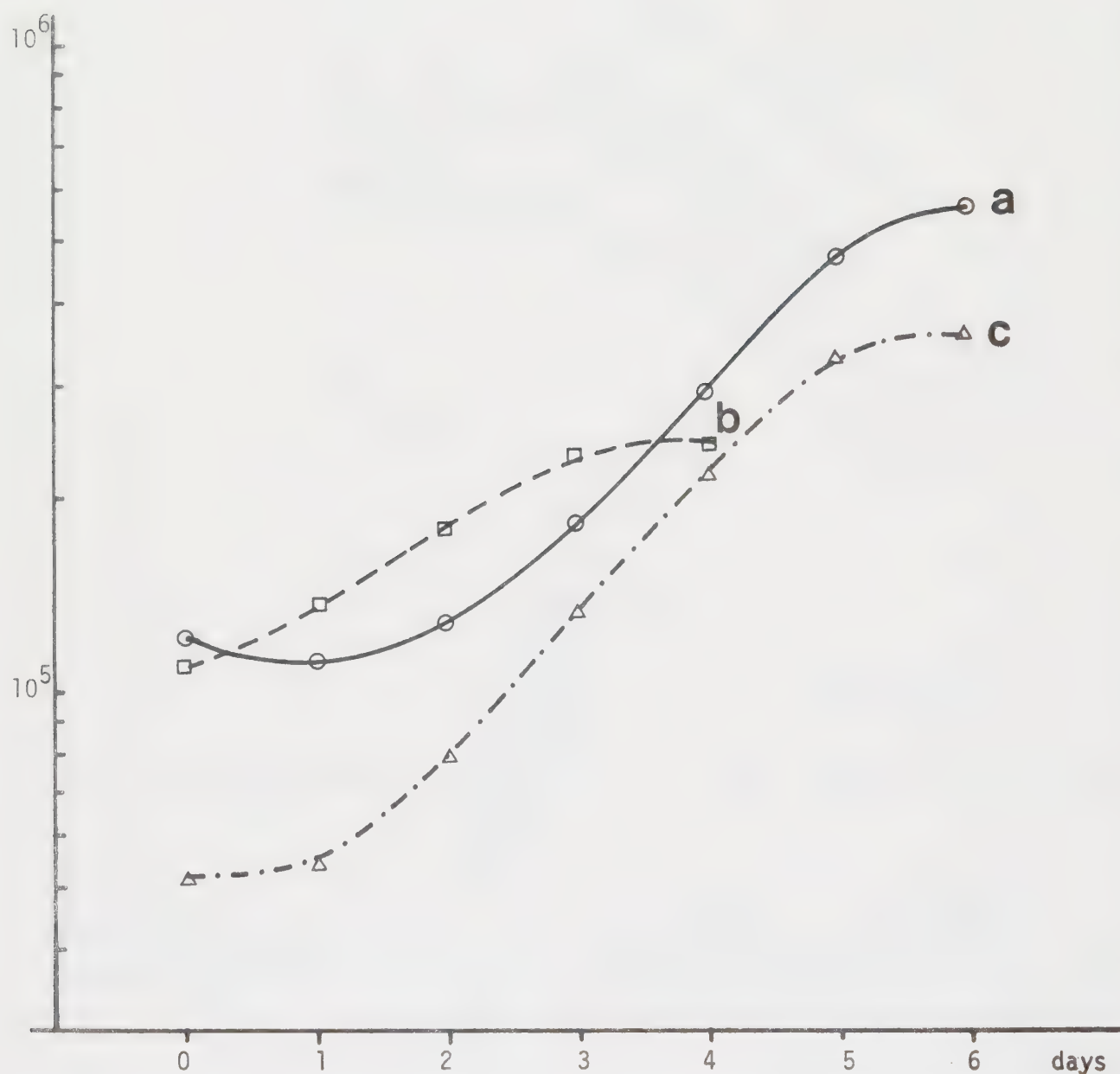


Fig. 4. Effect of trypsinization time and mechanical disintegration on growth of heart cells.

- a) ○—○ 18-day cells trypsinized for 30 min.
- b) □---□ 5-day cells trypsinized for 90 min.
- c) △-.-△ 12-day cells mechanically disintegrated.

cells/dish

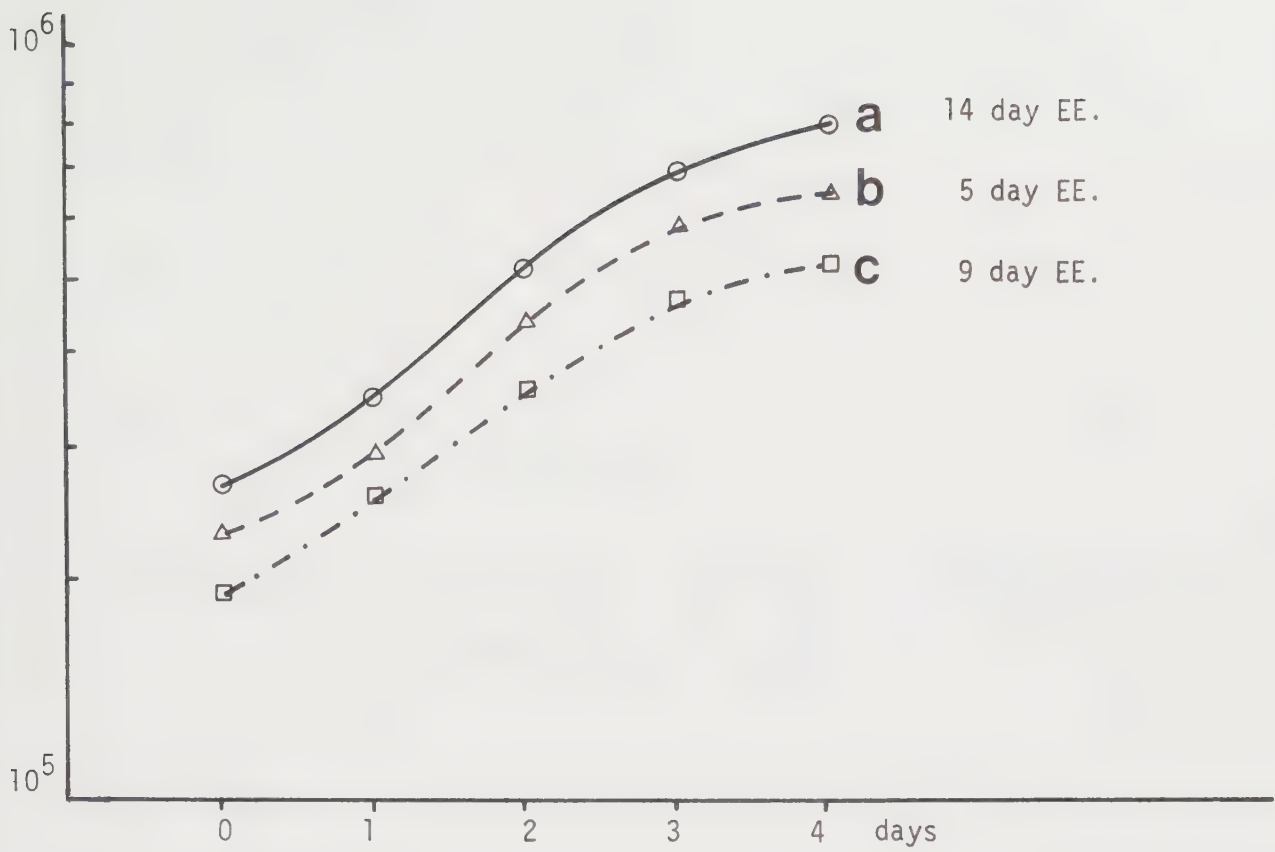


Fig. 5. Growth of 5-day heart cells in extracts of embryos from different ages.

- a) ○—○ in extract of 14-day embryos
- b) △---△ in extract of 5-day embryos
- c) □-.-□ in extract of 9-day embryos



CHAPTER 3

DIFFERENTIATION POTENTIALITIES OF CHICKEN HEART CELLS FROM DIFFERENT EMBRYONIC AGES IN CULTURE

RESULTS

The findings presented in Chapter 2 pose a fundamental question about the nature of the cultivated cells from different ages. Do they represent the same cells (4) but in different stages of differentiation or metabolic capabilities (54) and therefore have different inherent growth capacities? Or are they different cell-types (54) with different rates of proliferation (61, 17)? Cultures with high growth capacity might be the result of selection (64) of a particular undifferentiated and rapidly-growing cell (53).

Primary chicken heart cells placed in a medium containing serum supplemented with EE "growth medium" (84) soon start an active proliferation phase (20, 84, 4, 100) accompanied by the loss of differentiated properties (84). Accordingly, an active phase of growth (increase in mass) (86, 84, 4) and differentiation (4), but not of proliferation (100), starts if the cells are grown in a medium containing serum but deprived of EE "basal-medium" (84). This possibility of altering growth and differentiation (i.e. the characteristic behaviour of cells) by manipulating nutritional conditions has been the main object of the attempts to answer the above question about the nature of isolated chicken-heart cells from different embryonic ages.

One of the criteria on which the characterization of myocardial cells is based is the manifestation of structural and functional differentiation, i.e. cross-striation and beating of these cells during the culture period. Thus one of the aims of the present analysis was to find

a correlation between differentiation of the heart cells and their developmental age. Also additional parameters that can provide further information about the differentiation of the heart cells, such as rate of proliferation, effect of EE, spontaneous contraction, state of nucleus, effect of local cell densities, and evolution and deterioration of cross-striation, were examined.

1. The influence of developmental age of the cells on their structural differentiation.

Chapter 2 showed that the proliferation capacity of the heart cells in a growth medium increased with the increase in embryonic age (Fig. 1 and 2, Chapter 2). Moreover, it mentioned that the growth capacity was inversely correlated with the differentiation state of the myoblasts, i.e. the higher the embryonic age, the higher the growth capacity and the lower the differentiation (structural) properties. Further experiments performed to verify the findings presented in Chapter 2 showed that, in a growth medium composed of 10% EE + 20% serum + 70% Tyrode's BSS, the 18- and 12-day cells, at the end of the exponential proliferation phase, revealed far less frequency of structural differentiation than the 4-5-day cells (Fig. 1).

However, as Fig. 1 shows, structural differentiation at the end of or during the exponential growth phase is not a specific property possessed merely by the 4-5-day cells, but is a property characterizing the heart cells of all ages. Thus, corresponding values for percentage of cross-striated cells could be found when comparing the growth curves of cells from 5-, 12-, and 18-day cells, providing they exhibited a similar growth pattern, i.e. the higher frequency of differentiation is due to the lower proliferation activity irrespective of the cells' developmental age. Therefore the expression of differentiation practically depends on factors other than developmental age.

2. The correlation between proliferation of the cells and their degree of structural differentiation.

As already seen, the degree of differentiation correlates with the growth pattern of the cells in culture (Fig. 1). This is clearly demonstrated in the cultures that lack a substantial proliferation (Fig. 2). The replacement of EE (growth promoting factor) by a basal medium (30% serum +

70% Tyrode's BSS) enabled the cells to show high differentiation capacities irrespective of their embryonic age.

Electronmicrographs taken from ventricles of intact hearts from 5-, 12-, and 18-day embryos show that almost all the cells possess cross-striations. None the less, the cross-striation seems more abundant in 18-day cells and occupies most of the cytoplasmic space (Plates 1 and 2). Through the action of trypsin (61) or simply because of dissociation process when separating the cells (54), most of them lose this specific characteristic, although a few per cent still exhibit some extent of cross-striation (91, 61) (Plates 3 and 4). This lack of differentiation continues to persist after cultivating the cells for the first 24 h in the basal medium (Fig. 2). From this stage of the culture, depending on the quality of the medium, the cells will develop in one of the following ways: a) proliferate but not differentiate; b) differentiate but not proliferate; and c) proliferate and differentiate simultaneously (Fig. 1, 2, 3, and 8 show these tendencies). But the question arises whether there is an actual correlation between the degree of proliferation and the ability to differentiate, and if so, how does the proliferation affect the differentiation state of the cells? Analysis of the above-mentioned figures indicates that the appearance of differentiation during the culture period mainly depends on the slope of the peculiar growth curve, i.e. on the proliferation rate of the culture. It can be seen that cultures with a high growth rate that approach one generation cycle per day totally lack any differentiation. Moreover, it can also be seen that this tendency of lack of differentiation already commences at a growth rate of about 40% (1.4) per day. In some instances (Fig. 1a₃-1b₂-3b₁-3b₃) already at growth rates around 40%, there is no exhibition of cross-striation. On the other hand, instances could be found where cells with growth rates of similar orders (Fig. 1a₂, 1b₁) or even higher (Fig. 3a₁) showed considerable differentiation. The explanation of this contradiction could be traced to a time factor, i.e. the behaviour depended on the duration of the growth rate in question. If the proliferation rate of a culture was 1.46 (Fig. 1a₂-1b₁) or even 1.58 (Fig. 3a₁) for a short time one day, for instance, at the beginning of the culture, and it then decreased or stopped for the remainder of the culture period, the capacity of differentiation would not be entirely lost. But if a growth rate of similar magnitude was manifested over a longer period (Fig. 1a₃-1b₂-3b₁), for instance, 3-4 days, the manifestation of differentiation would be almost completely suppressed.

It could be expected that, by cultivating the cells in a medium that did not promote proliferation (basal medium), the results would lead to a higher percentage of differentiated cells. But in practice, this was realized only in occasional cultures. The variety of results obtained is as follows: (Fig. 2)

- a) appearance of a certain percentage of cross-striated cells is not a constant phenomenon, even in experiments performed under similar conditions.
- b) cell populations representing the actual embryonic ages in proper conditions are able to produce cultures with a high percentage of differentiated cells.
- c) the emergence of differentiation is gradual and increases along with the culture period, reaching maximum values in the 3rd-5th day of culture; it then gradually decreases. The decrease, being accompanied with dissolution of cross-striation, parallels the fat accumulation in the cytoplasmic space (Plates 12 and 13).

After it was noted that cell proliferation antagonizes cell differentiation, the question arose: How can removal of the proliferation factor from the culture medium affect cell differentiation after an active proliferation phase? Practically, this implies analysis if actual redifferentiation takes place when the growth medium is changed to basal medium. Thus cells of different ages were cultivated in growth medium for 4 days to achieve maximum growth; the medium was then replaced every third day by basal medium. The results (Fig. 3) show that the reversibility of the differentiation state depends on the intensity of the proliferation in the previous growth phase. At a growth rate of 1.9 times/day (Fig. 3c₁) or by a longer time action of a milder growth rate, such as 1.46 times/day (Fig. 3b₃), the reversibility of the differentiation was almost entirely lost, even when the cells were cultivated in basal medium for an additional 7 days. However, under these conditions, the differentiation did not reappear; instead, the cells accumulated fat droplets and eventually died (Plate 20). Interestingly, those cultures whose rate of proliferation was considerably lower in the previous growth phase were, in the presence of basal medium, only partly redifferentiated (Fig. 3b₁ and 3c₃). As far as 12- and 18-day cells are concerned, the reappearance of the percentage of cross-striated cells was gradual. Surprisingly, the highest values were about 20-40%. These appeared on the 4th-5th day after replacement of the growth medium.

Prolongation of the culture period up to 12 additional days in basal medium did not increase the percentage of cross-striated cells; instead, they subsequently decreased. The differentiation state of the cells also gradually deteriorated, paralleled by the accumulation of fat droplets in the sarcoplasmatic space (Plate 20); the cells finally dying. On the other hand, a definite conclusion was difficult to draw concerning the 5-day cells' ability to reverse the differentiation, as a high percentage (70-80%) of differentiated cells was observed already one or two days after replacement of the growth medium. Fig. 3a shows these two specific instances. However, it is probable that as these cells did not show a remarkable proliferation activity during the growth phase, they could manifest a population of high percentage differentiated cells already one or two days after replacement of the growth medium. This suggests that 5-day cells in these conditions did not lose their original structural differentiation during the growth phase, even in the presence of growth medium containing 10% EE (Fig. 1a₁-1a₂).

3. The effect of EE on growth and differentiation.

EE is the factor that causes cell proliferation, and there is antagonism between cell proliferation and differentiation. Thus it was essential to study the effect of EE per se on cell differentiation. One series of experiments tested various concentrations of EE on cells of different ages. The results shown in Fig. 4 indicate that: a) The growth is concentration dependent; i.e. the higher the concentration of EE, the higher the growth rate in each age, and the lower the % of d.c., but it is not quantity dependent. For instance, the 5- and 18-day cells grow to different levels in the same concentration of EE. Thus the amount of growth is determined not by the amount of EE present in the medium, but by the actual growth capacity of the cells from different ages. b) A gradual antagonism exist between EE concentration and cell differentiation. However, the minimum concentration of EE needed for a total antagonistic effect differs for cells of different ages, i.e. about 5% for 18-day cells, 10% for 12-day cells, and 20% for 5-day cells. On the other hand, in a concentration of EE as low as 2.5%, the 18-day cells barely show differentiation whereas, in similar EE concentration, the 5-, and 12-day cells demonstrated populations of 20-30% cross-striated cells. Thus, concerning cellular proliferation and differentiation, the findings suggest that the EE might not have a similar effect on cells of different ages.

To test this further, another series of experiments were performed where the EE was gradually denatured; 5 ml EE was heated in a water bath at 56°C, 64°C, and 80°C for 30 min and at 100°C for 5 min, and after 30 min centrifugation (3000 rev/min), the supernatant was incorporated into the culture medium as ordinary 10% concentration. This was done to reduce the antagonistic effect of the proliferation on differentiation, because, in some preliminary experiments, it was found that denaturation reduced the ability of EE to support proliferation. However, some amounts of proteins were also precipitated after the denaturation process (Fig. X, Chapter 1). The process was also intended to destroy the specificity of the EE, if there is such a specificity.

The results shown in Fig. 5 show that

- a) The ability of EE to support proliferation decreases gradually with increase in the denaturation temperature.
- b) The normal EE supports the multiplication of about one additional generation cycle when dealing with cells of high growth capacity.
- c) The 20-day cells (in this particular case cells from a higher embryonic age were used to increase the growth capacity) can use the NEE much more effectively. Unlike the 12-day cells which grow to the same extent in both NEE or EE denatured at 56°C, the 20-day cells continue their proliferation in NEE far beyond as in DEE at 56°C. The cells from higher ages (18-20 days) are apparently able to utilize the HMW fraction of the EE, which is precipitated if subjected to heat denaturation (Fig. X, Chapter 1). This might also explain the high residual growth energy (73, 15) possessed by cells of higher ages, as found in the present study (Chapter 2).
- d) Concerning the 12-day cells, Fig. 5 shows that there is a gradual antagonism between the rate of proliferation and the percentage of differentiated cells. Also there is a direct correlation between the percentage of differentiated cells and the increase in denaturation temperatures.

In 20-day cells, the percentage of cross-striated cells is generally low. Furthermore, the growth-rates of both 20-, and 12-day cells in denatured embryo-extracts are somewhat similar, but the 20-day cells lack, or have a much lower percentage of cross-striated cells.

These findings suggest either that the 18-20-day cells are to some extent irreversibly de-differentiated after the disintegration process,

or that the presence of the EE, even in denatured form, has still antidifferentiation activity, or both.

The effect of "contact inhibition" (1, 2) on a cell culture reaching monolayer level is to inhibit the movement of the cells and eventually their proliferation: "contact inhibition of growth" (12, 52, 71). Therefore, the presumption that high initial cell concentration might cause a rapid monolayer formation without a substantial proliferation led to the setting up of cultures with high initial cell concentrations in growth medium (i.e. in the presence of 10% EE), in basal medium (0% EE), and in basal medium supplemented with 2% EE. The results obtained (Fig. 6, 7, and 8) were the following:

a) In a culture with a high initial cell concentration and complete lack of proliferation (Fig. 7), the high percentage of EE (10%) results in the appearance of a low percentage of cross-striated cells (only 5-10%). Conversely, under the same conditions but in a lower percentage of EE (2%), about 50% of the cells were gradually cross-striated. Similar results are seen in Fig. 6, where cells in 10% and 0% of EE are compared. In the latter, a slightly higher growth rate, 1.30 to 1.12, does not seem to have any substantial effect, as this slight growth rate takes place only during one day; moreover, a comparison of Fig. 6 with Fig. 7, shows that the percentage of cross-striated cells is even higher in the former despite this particular growth-rate.

b) The above result was further confirmed when the cells were first grown in 10%, 5%, and 2.5% EE for 3 days, and the medium was then replaced by basal medium. This was done to see to what extent the concentration of EE affects the cells during the first 3 days, and if any injurious change caused by EE is reversible.

Fig. 8 shows that when the cells are grown in 2.5 per cent EE, as much as 70-85% of the cells are differentiated after two changes of the basal medium. Conversely, growing the cells in 5 per cent EE as much as 60% of the cells are differentiated, despite the growth rate being identical with that in the former case (1.08/day). Concerning the effect of 10 per cent EE, although a growth rate of 1.28 was recorded during the first day of culture, it cannot of itself account for the relatively poor percentage (10%) of cross-striated cells, as during the remainder of the culture period, the growth rate was of the same order as it was for 2.5

and 5 per cent EE. Therefore it is obvious that a high percentage EE (10%) has an antidifferentiation activity, although it is not necessarily accompanied by cellular proliferation, and the injury (Plates 14 and 15) it causes is almost irreversible

c) A low growth rate of about 1.10/day does not suppress -- it even enhances -- the formation of a population of a high percentage of cross-striated cells (Fig. 8), providing the cells are grown in basal medium or basal medium supplemented with low concentration of EE (1-2%). In these conditions, more than 80% of the cytoplasm of the tested 12-day cells were completely differentiated (Plate 21).

4. The relation between the structural and the functional (beating) differentiation.

Beating of isolated cells from heart muscle in culture is an additional indication that points to the myogenic origin of these cells. As Rinaldini suggests (84), the beating and even the inactive form (non-beating) of heart cells in culture might represent two aspects of the same cell. The question is, whether beating and nonbeating cells isolated from embryonic heart are actually of the same myogenic origin. In the following, the beating property is assessed in respect of density of cells and of degree of structural differentiation during the culture period and previous to fixation. The results given in Fig. 9 and 10 conclude these observations. As no marked differences were observed among the cells of various ages, the values are given irrespective of the embryonic age of the cells. Thus Fig. 10 shows: a) there is no correlation between the density of cells in a given area (Petri-dish) and the degree of beating exhibited by these cells. It can easily be found (Fig. 10) that the cells can vary their degree of spontaneous contraction in cell densities ranging from 5×10^4 cells/dish to 9×10^5 cells/dish (approx. 50-900 cells/mm²). However, a higher frequency of beating activity that appears in optimum cell densities (300-600 cells/mm²) is a result of the cells' optimum physical contacts leading to local synchronous pulsation (84, 32, 33, 91). Parallely, it can be seen that similar cell densities under the same conditions do not show any pulsating activity. In this connection, it should be noted that occasional beating of single cells from the time of counting in the Bürker chamber after the isolation of the cells and during the first days of culture period frequently occurs. Thus these findings suggest that the

ability of spontaneous contraction is a myogenic property of the single cells (32) and that its expression is modified by a variety of factors (20).

b) As Fig. 9 shows, there is no correlation between the extent of beating of a cell culture and its percentage of cross-striated cells. However, often a higher frequency of non-beating cells is found in undifferentiated cultures; moreover, cultures with a high percentage of cross-striated cells normally show a high degree of beating. Two other phenomena, however, are commonly observed during the cultivation of embryonic heart cells. Occasionally, a population of cross-striated cells does not show any spontaneous contraction. Fig. 9 shows that cultures with a population of up to 50% cross-striated cells might not reveal any pulsating activity in some particular stage of the culture's life (17). On the other hand, spontaneous contraction of the cell cultures almost deprived of a significant percentage of cross-striation (Fig. 9) is a common phenomenon. Occasionally, cultures were found to be beating in various degrees at the moment before fixation, but after staining, often less than 1 to 2% cross-striated cells in a rather incomplete state could be observed. The former case, when the cells are differentiated but do not beat, is understandable, as various factors, e.g., temperature, pH, salts, substrates, metabolic inhibitors (33), and serum, K^+ concentration (20) can affect the manifestation of this attribute. But the observation found in the latter case - that cells can beat without visible cross-striation - was rather surprising. The finding is of interest, as it provides evidence that a visible structural differentiation of heart cells in culture is not a prerequisite for contraction (4). Apparently, this function might be achieved on other levels of structural organization and without the arrangement of contractile proteins in a perfect pattern of cross-striation (45, 46). Further examinations of the cytoplasmic structure of these cultures revealed that in many instances the degree of beating was correlated to the presence of the myofibrillation of the cells which were not necessarily cross-striated (Plate 8). Moreover, in other cases, a visible myofibrillation was not a prerequisite for attaining spontaneous contraction, i.e. this function is possible even in less organized cytoplasmic structures.

Additional remarkable results obtained regarding the beating property, indicate

a) An actual correlation between structural differentiation and the rate of beating, for instance, in cultures, where the rate approaches 100

contractions a minute, the cells are completely cross-striated.

b) A gradual loss of the ability to beat with increase of EE concentration in culture medium. Thus, in a concentration of 20 per cent EE, the ability of spontaneous contraction is much reduced. This inhibitory effect of the EE on beating is probably due to its antidifferentiation activity, possibly coming from the destruction of contractile and even structural proteins (Plate 15).

c) A tendency to varying beating during the culture period. Usually a low degree of beating is seen from 0-1 day probably as a result of the scarcity of cellular contact. It then rises to maximum on the 2-4 days, and subsequently decreases in the rest of the culture period.

d) The cells continue to beat in proliferating cultures, but the degree of beating reduces or even ceases in high rate proliferating cultures.

e) There is no gradual decrease in the degree of beating in cells from 5- to 18-day embryos (20), but variations in the frequency of beating have been recorded from culture to culture.

5. The relation between morphological appearance of the nucleus and cytoplasmic differentiation

Irrespective of embryonic age or morphology of "cell types" in culture, the morphological appearance of the nucleus, as observed in this study, was found to be a good indication of the myogenic character of cells. Thus a rather small, spherical, and densely stained nucleus possessing predominantly one concentrated nucleolus belonged to a cell with a well cross-striated cytoplasm (Plates 7, 11, 12, 21, 23, 24, 25, 28). On the other hand, a relatively large (35, 36, 29) to very large and not densely stained nucleus with more than two nucleoli, or scattered chromatin, was an indication of actively proliferating cells lacking cytoplasmic differentiation (Plates 14, 15, 16). Such descriptions apply especially to cells grown in EE (in all concentrations), particularly in high concentrations in the order of 10-20%. This effect is further intensified in low cell densities and in spread cells (Plate 18). Furthermore, a large nucleus is frequent in cells of prolonged cultivation (Plate 19) and in cells of higher ages (Plate 17). It is particularly interesting that the former morphological

features of the nucleus are occasionally correlated with cytoplasmic structures other than a defined pattern of cross-striation, i.e. a fibrous or a homogeneously granulated cytoplasm was often found in such cells (Fig. 11 and Plate 8). However, the previous findings, that spontaneous contraction could be carried out in similar cytoplasmic structures, suggest that the myocardial cells in culture are able to exist at various levels of structural organization and still perform their specific function (beating).

6. The evolution and deterioration (dissolution) of the cross-striation in the course of the embryonic myocardial cell culture

The evolution of differentiation during the culture period takes place in two ways:

- 1) Increase in the number of differentiated cells in a certain cell population.
- 2) Development of the cross-striation in the single cells.

Thus a certain condition supporting cell differentiation would be regarded as optimum when it leads to a high percentage of differentiated cells in a population in which the cells are completely cross-striated (Plate 7). However, in the present study for the calculation of percentage of differentiation, any cell that showed signs of cross-striation, to any extent, was taken into account as a potentially differentiated cell. Therefore the presentation of, for instance, 70% differentiated cells in a particular case does not indicate that the cells are completely cross-striated (Fig. 11₂ and 11₆); on the other hand, a population of, for instance, 20% differentiated cells might represent a case in which the cells are completely cross-striated (Fig. 11₄). Thus, although the method of calculating the percentage of differentiated cells could give proofs of the cells' myogenic origin, it does not describe the appearance and the sequence of the development of cross-striation during the culture period.

As previously mentioned, the action of tissue disintegration led to heart cells completely or largely losing their cross-striation (Fig. 2 and Plates 3 and 4), and the reappearance of cross-striation depended mainly on the quality of the medium. Thus, in the presence of basal medium, either from the beginning of the culture or after replacement of a growth medium, the cross-striation started to reappear. This reappearance in cells grown in the basal medium directly after disintegration (Fig. 2) was different from cells that first experienced a period of proliferation and then had a

replacement of the basal medium (Fig. 3). In the former instance, either a high percentage of completely cross-striated cells emerged rapidly during a short period (1-3 days) (in an adequate environment, for instance, with the addition of 2 per cent EE or 16 mg/ml bovine albumin to the basal medium) (Plate 7) or irrespective of percentage, striations of rather similar qualities appeared during the first days of the culture period, followed by deterioration (Plate 13). However, in the latter event, i.e. when the cells were allowed to experience a period of mild proliferation and then received basal medium, the development of cross-striation appeared more gradually and distinctly. The sequence of the development of cross-striation is shown in Plates 8, 9, 10, and 11 and in Fig. 11. This schematic drawing does not show the succession of events that take place in merely one single experiment, but is rather a synthesis of results obtained from many single observations. Thus a homogeneous or homogeneously granulated or myofibrillated cytoplasm with a typical nucleus (see paragraph 5) indicated a myogenic cell lacking a visible differentiation (Fig. 11₁). Cells with such organization could be observed throughout the culture period. The changes that occurred after this stage (41) are:

- 1) Appearance of thin, dark, band-units running longitudinally along a few myofibres or in the uniform cytoplasm (Fig. 11-2 and Plate 9). This occurs about one day after the replacement of the growth medium (Fig. 2).
- 2) Thickening of these units (A bands) (4) and emergence of new fibres containing such bands (Fig. 11-3 and Plate 10) after 1-2 additional days.
- 3) Occupation of almost all of the cytoplasmic space by cross-striated fibres with distinct A, H, and I bands (Fig. 11-4 and Plate 11) (the Z band was rather obscured) was completed 4-5 days after replacement of the growth medium. Similar events occurred in cells lacking visible myofibres, the last step being the association of the lateral bands that gave rise to a complete cross-striated cell (Fig. 11-4 and Plate 11).
- 4) From this stage on, the gradual decomposition of this structural organization appears. Consequently, the A band becomes increasingly thinner from the H band outwards. This ultimately leads to a disorderly dissolution of the remaining A band (Fig. 11-5 and 6 and Plates 12 and 13).

DISCUSSION AND CONCLUSIONS

The modifications in phenotypic expression of the cultured heart cells exhibited in the present study were found to be a consequence of variations in the culture conditions, as found also by DeHaan (20). None the less, all the parameters tested indicated genotypic stability of these cells. Thus cultures of more than 80% differentiated cells were observed in the three respective ages, which suggests that proper conditions that promote differentiation enable the cells of all developmental ages involved to show their myogenic potentialities. Thus the change in phenotypic expression was caused primarily by the antagonistic effect of proliferation on visible differentiation. These observations indicate that multiplication in isolated cultured cells antagonizes "re-differentiation", but is not necessarily the direct cause of primary cell change (54). The differentiated cells in the intact heart were, by a disintegration process, brought into a suspension of cells, and almost all lacked a visible cross-striation. The loss of striation could occur, as Mark et al. (61) suggest, by digestive action of trypsin on myofibrillar proteins, or the alteration is a response to the artifacts of culture conditions (54). A nutritional deficiency was suggested by Cahn et al. (14) to account for the loss of original differentiation in retinal pigmented cells. However, disregarding the effect of the EE, the rate of proliferation determined whether or not a culture would structurally re-differentiate. Furthermore, this finding applied to all cultures irrespective of the developmental age of the cells. These findings agree with the definition of "de-differentiation" formulated by Strangeways in 1924 as cited by Waymouth in (100) that de-differentiation implies the latency, but not the loss of specific potentialities. In this connection also the term "modulation" calls for comment. "Modulation" means loss of some actual functions without change of potencies. This term, as applied by Weiss and cited also by Waymouth in (100), is particularly adequate when applied to cells in basal medium, in which the specific potentialities in a majority of cells reappeared after a relatively short time (3 days). Modulation still applied where the cells experienced a period of "mild" proliferation, as removal of the growth factor from the medium enabled the cells to re-exhibit their specific structure. However, a vigorous rate of proliferation completely inhibited the re-exhibition of structural specificity, suggesting irreversible alterations in structural proteins; thus an irreversible re-differentiation. Agrell (4), studying heart cells in a similar system, considered the

de-differentiation-differentiation to be a modulative modification. Stockdale and Holtzer (93) found that myotubes, formed in vitro from skeletal muscle cells, and also myoblasts, from somites of 3-4 day embryos synthesizing contractile proteins in vivo, do not synthesize DNA for mitotic division. On the other hand, Manasek (60), examining embryonic muscle cells in vivo, demonstrated mitosis among the cells containing myofibre and concluded that the increase in muscle cell number in developing heart is the result of division in the general population of developing muscle cells. Similar results were reported by Weinstein et al. (101), who examined 4-5 day hearts in vivo, and by Chacko (17) in clones of 5 day cells in vitro. DeHaan (20) found that heart cells in vitro, contracting and having contractile myofibres, can in fact divide. Goldstein et al. (27) stated also that the presence of contractile protein in heart cells in vitro does not inhibit either DNA synthesis or mitosis. Division of heart cells both in vivo and in vitro was also reported by Polinger (80).

Rough estimates of cell-yield in the present study indicate that the average increase in cell number in the chick embryo heart from day 5 to day 18 is from 2×10^5 to 10^7 , i.e. a 50-fold increase in cell number (the intermediate values being roughly 1×10^6 /7-day heart and 3.5×10^6 /12-day heart). Thus this increase very probably results from the division of differentiated cells, as the 5 day cells in vivo are fully differentiated (101); and as DeHaan has found (20), the proportion of muscle cells in vitro is invariable in all ages. However, the "antagonism" found in the present study emphasizes the significance of proliferation rate and the duration of time in which it operates on re-differentiation process. Moreover, the re-differentiation initiates from an undifferentiated, i.e. not-visibly differentiated (4) state. These findings agree with the idea that cellular multiplication and cellular differentiation are distinct processes (Bloom and Willmer as cited by Waymouth in (100) and Agrell in (4)). Moreover, the more vigorous the proliferation, the less the manifestation of specific properties in a cell population (Waymouth (100). Eagle (24), with relatively few exceptions, extended the propagation of cells in tissue culture accompanied by a loss of their initial in vivo differentiation. Holtzer (44) found that, if an embryonic cell population of cartilage is forced to divide in monolayer five times in close succession, the resulting progeny cease to synthesize chondroitin sulphate, but if the cells divide only twice, their progeny still retain their synthesizing capacity. However, by cloning retinal pigmented

cells, Cahn et al. (14) found that pigmented cells which subcloned four times and underwent over 50 times cell divisions (28-32 h generation time) remained pigmented. Konigsberg (53), by culturing embryonic skeletal muscle, also found that, despite the culture conditions which would have promoted rapid growth (18-20 h doubling time, for 3-4 days), the cultures differentiated. Thus, from these observations, it is apparent that there could be variations in different cell systems and in different culture conditions which, on differentiation, respond differently to the antagonistic effect of proliferation. For instance, in the former case, the retinal pigmented cells were grown in a medium containing 2 per cent low molecular weight fraction of EE, but if grown in 2 per cent high molecular weight fraction, the generation time would have been shorter (16-20 h) and no pigmentation would have occurred; thus the effect of the high molecular weight fraction of EE was very crucial in preventing differentiation. In the system used in the present study, 2 per cent whole EE promoted re-differentiation in a rather low growthrate (1.10/day). Concerning the latter case, Konigsberg (53) suggested that the multinuclear condition of skeletal muscle cell explained this contradiction, i.e. the formation of myofibril in multinuclear cell might be postmitotic.

A secondary inhibition of phenotypic expression was found to correlate with the presence of EE in culture medium, regardless of its proliferative effect. The EE was found to have two separate effects: growth promoting, and antidifferentiative. Furthermore, the proliferative activity of EE is concentration dependent (but not quantity dependent) and is heat-labile. The literature has similar findings concerning proliferative and antidifferentiative effect of EE. Thus Harris (39) found that the logarithmic growth rate of skeletal muscle cells is proportional to the concentration of EE. Cahn et al. (14), summarizing several studies, stated that in most studies in which de-differentiation was reported fairly high amounts of EE were used and in the presence of large concentrations of high molecular weight fraction of EE, the cells are inhibited from expressing their differentiation. In their study, Cahn et al. (14) showed that, in the presence of high molecular weight fraction of Sephadex G-25 separated EE, the retinal pigmented cells lost their original pigmentation whereas in the presence of the low molecular weight, they did not. Similar results were obtained by Coon and Cahn (19) in clones of cartilage and pigmented retina cells. Kutsky (55), Harris and Kutsky (38), and Kutsky and Harris (56) tried to

trace the promoting effect of EE on proliferation to the nucleoprotein fraction of EE. Some positive results were obtained concerning the out-growth, growth, and morphology in the presence of the nucleoprotein fraction of EE, but the nature of the active factor was not ascertained (55). Nor was any correlation found between nucleic acid content and growth promoting effect of EE (55, 56). On the other hand, Winik et al. (103) demonstrated that actual utilization of amino-acids, peptides, and proteins of EE takes place in cultures of embryonic heart cells. These findings, in addition to those obtained in the present study, suggest that EE is a source of nutrition both for multiplication purposes and for differentiation (in low concentrations, e.g., 2%) because solely in serum, there is no multiplication, and the differentiation is incomplete. Moreover, this contribution is made primarily by all the low molecular weight substances (metabolites) present in EE. Furthermore, the high molecular weight substances (> 5000 MW (14)) might have two additional functions:

- 1) to supply additional nutritional means and
- 2) to contribute antidifferentiation effect.

The former suggestion was shown to be probable, as cells with higher growth capacity could use this fraction (as a result of higher, metabolic capabilities or permeability) if the EE was not denatured (Fig. 5), i.e. if the high molecular weight was not precipitated (Fig. X, Chapter 1). The latter suggestion too was shown to be probable (14, 19), but the mechanism of action is rather obscure. Cahn and Cahn (14) stated, "the mode of action of substances inhibiting expression of differentiation is unknown", but they suggest that it might be due to both spreading activity of high molecular substances and loss of material from the cell to the medium by directly increasing the permeability. However, as in the present study, the antidifferentiation effect of EE was found to be much more pronounced in cells of higher than lower ages. This might reflect changes in the surface of embryonic cells correlated with differentiation and maturation (Kleinschuster and Moscona (51)) and thus the permeability caused by this fraction and the intracellular changes provoked by it might be different in cells of different ages. None the less, the effect of EE, to some extent, does not exclude enzymatic reaction, as the effect is concentration dependent and heat labile. In addition, an interaction between the proteolytic effect of the EE and serum as an antiproteolytic factor (90) is also possible, as serum inhibit this effect in

low EE concentration (2.5% EE + 27.5% serum), in which the 12-day cells differentiate but not in higher concentrations of EE. This effect also might account for the lysis of the cells when grown only in EE without the presence of serum (Chapter 1, page 14 and Fig. III). Thus the high molecular weight fraction possibly contains proteolytic enzymes that might act on the cell membrane or even enter into the cells, causing decomposition of structural proteins and resultant de-differentiation. The latter argument was advanced by Mark et al. (61) to account for the digestion of myofibrillar proteins in newly trypsinized heart cells. However, the preferential permeability of the cells from higher ages to this fraction might explain the lack of re-differentiation observed in these cells.

The myogenic character of cultivated cells was indicated by spontaneous contraction manifested by single cells (84, 32) independent of cell density and, to a great extent, irrespective of visible cross-striation. On the other hand, both these factors were found to correlate with an improvement in functional activity, one by increasing the physical contact among the cells (32) leading to a local synchronous activity (91); the other by increasing the pulsation rate. In addition, no direct correlation was found between the embryonic age of the cells and the ability of beating when the cells were grown in basal medium. However, the cells of all ages could beat equally well in growth medium during the mitotic phase, although the degree of beating was reduced in high growth rates and high EE concentrations. Rinaldini (84) stated that rapidly growing cells might not show functional differentiation. However, DeHaan (20) found that heart cells can beat in a static state and also when entering a period of rapid mitosis. Mark et al. (61) also stated that heart muscle cells might undergo mitosis while contracting. Despite the dissimilarity in the criterion evaluating beating, the results of the present study disagree with those found by DeHaan (20) that the percentage of actively beating M-cells decreased with increasing embryonic age, whereas their proportion is unchanged in all ages. A well cross-striated pattern with distinct bands, as shown by electronmicrographs, was observed in the in vivo heart cells of all ages (Plates 1 and 2). Hibbs (41), who examined developing cardiac muscle in vivo, demonstrated that, before 30 h incubation, the myofibrils were present, and already after 60 h incubation, all bands were formed. None the less, a well cross-striated muscle fibre is the basis for the mechanism of contraction in vivo (Huxley 45, 46). In the present study, the in vitro results, which showed a lack of a substantial

percentage of cross-striated cells in a beating culture, do not in all cases agree with such a mechanism of contraction. In support of this incompatibility, Agrell (4) also found that a cross-striation does not seem to be an absolute prerequisite for the contraction in heart-cell cultures. Smith et al. (91) also observed that, of the few heart cells which exhibited spontaneous contractility early in culture (2-3 hours) none appeared to possess cross-striation. On the other hand, Rumery (88, 89) observed that, unless a myofibril had acquired striation, no contraction could be observed. And Harary (32) stated that the process of de-differentiation is apparently accompanied by loss of striation and cessation of beating. This agrees with the irreversible de-differentiation observed in the present study. However, in the presence of cross-striated cells, spontaneous contraction was always manifested (except temporarily cessation, independent of cross-striation). Furthermore, this correlation was highly accentuated in cultures with relatively high rate of contraction ($> 100/\text{min}$). DeHaan (20) summarized many studies, concluding that "the spontaneity of the heart is dependent upon the rhythmic generation of bioelectric potentials by specific pacemaker cells." In the same article, he summarizes the in vitro results obtained by various authors, in which estimations of the proportion of pacemakers in the total population of cells ranged between 2% (32) and 88% (61). Concerning the cultures in the present study, in which contractile activity was observed without a significant number of cross-striated cells (1-2%), there are still objectives to be considered, particularly if we suppose that a few actively contracting cells (leading cells) (32, 33) initiated the wave of the contraction, which was then transmitted through the passively contracting cells ("following" cells):

- 1) The leading cells, as found in the present study, were incompletely striated (merely composed of A bands); this feature cannot be subjected to the "sliding-model" of contraction proposed by Huxley (45, 46).
- 2) The "followers" contract by themselves.
- 3) In proper conditions, more than 80% of cells in a population are completely cross-striated and beating, thus indicating that passively contracting cells too are myogenic and lack the pattern of striation, but have contractile proteins possibly in a different form of organization, for instance, as seen in the case of un-striated myofibres.

In this connection, Harary et al. (32) found the level of actomyosin in beating cells to be comparable to that of the intact heart, and they suggest that, possibly by the use of electronmicroscope or specific fluorescent antibodies, the unseen striation might be visualized. Furthermore, Smith et al. (91) suggested that the functional differentiation of un-differentiated cells is retained at a submicroscopical level. Thus these predictions were confirmed by Chacko (17), who observed that contractile myocardial cells that did not reveal cross-striated myofibrils with phase microscopy were found to contain striated and un-striated fibrils when they were glycerinated and treated with labelled antimyosin.

Morphological variations in the heart-cell cultures were distinguished by various authors. DeHaan (20) remarked that, after 24 h in culture, there were mainly two morphological cell types: M-cells resembling myoblasts of skeletal muscle (comprising just over 50% of the population), and F-cells that usually have the properties of typical fibroblasts (comprising 40-50% of the population). In addition, intermediate cells having morphological characteristics of both M- and F-cells could be seen (less than 1% of the total population). Furthermore, he stated that, in all developmental stages, the proportion of M-cells is rather constant, the values for 4-day hearts being 53.5% M- and 46% F-cells, and for 18-day hearts being 54.5% M- and 46% F-cells. A similar classification was also reported by Polinger (79), but she added that M-cells contain glycogen (gly⁺) and myofibrils, whereas most of the F-cells do not contain glycogen (gly⁻), and no F-cells contain myofibrils. Moreover, she concluded that the gly⁻ cells apparently derive from the epicardium, the endocardium, and the endothelial lining of blood vessels. However, Meyer et al. (64) reported that many muscle cells could be found among the F-like-cells, as they were seen to contain myofibrils when examined under the electronmicroscope. Also, regarding the opinions about the heart-muscle cells in vitro, Meyer et al. (64) concluded that possibly a de-differentiation process takes place, but not a selection of F-cells, owing to the degeneration of muscle and survival of fibroblast cells. Rinaldini (84) stated that morphologically there is a whole gradient in the heart-cell cultures from flattened and transparent "fibroblast" to the thick elongated myoblast; he suggested that the beating myoblast and inactive fibroblast might be two aspects of the same cell. DeHaan (20) even suggested that these two morphological forms might represent cells in their division cycle or their response to the conditions of culture. However, Chacko (17)

reported that beating cells of both morphological types, i.e. F- and M-cells, were observed in cultures established from atria and ventricles. Eventually, Smith et al. (91), because they observed F-cells demonstrating spontaneous activity, disagreed with the morphological criterion as a means of cell classification. In their tests, they observed that all cell types (spindle shaped, rectangular, and irregular shaped) were capable of spontaneous activity. In addition, they observed that the F-cells during the culture period gradually became less common, so that, by the 8-10-day stage, nearly all the cells in a given culture appeared by morphological characteristics to be myoblasts; finally, they concluded that the F-cells are in fact myocardial cells in the process of adjustment to the in vitro conditions. The results obtained in the present study largely agree with these observations. However, it can be added that in the present study more than 80% of cells including all cell types were also structurally differentiated (Plates 7 and 21-28).

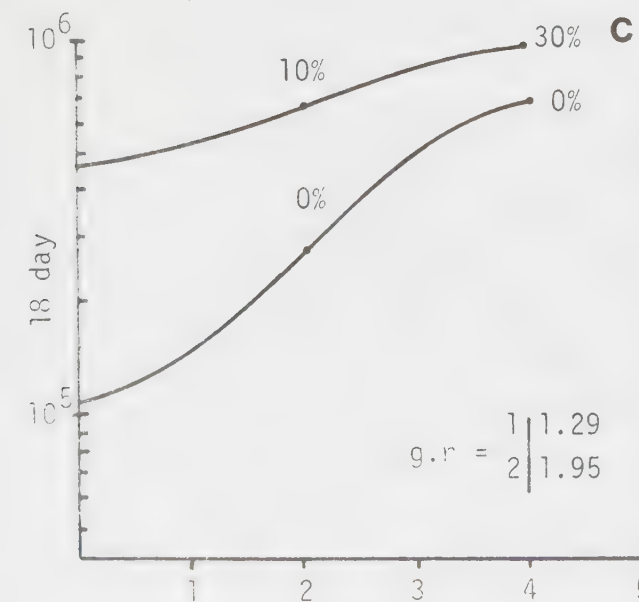
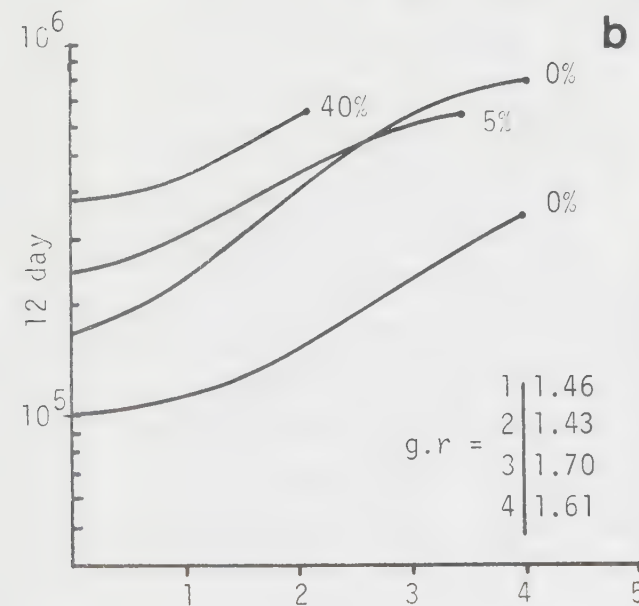
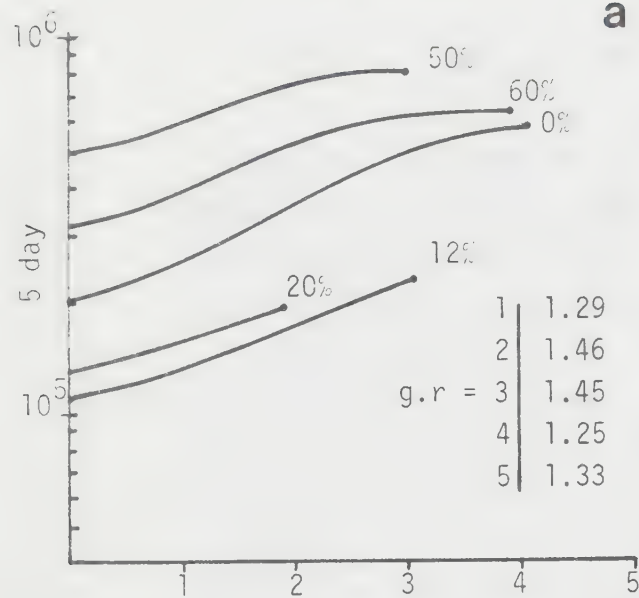
On the other hand, an additional cell-type was reported to be present in heart-cell cultures by Mark et al. (61) and Chacko (17). Thus, Mark et al. (61), in cultures of newborn rat heart-cells, found two types of cells morphologically distinct: 1) muscle cells, and 2) endothelioid cells, which have clear cytoplasm, larger nucleus than M-cells, and contain several nucleoli, but do not contain myofibrils or beat, and are very mobile and change their shape. Also the endothelioid cells divide more rapidly than M-cells. Furthermore, they concluded that most probably they are endothelial cells from the many capillaries throughout the heart muscle. They added that the proportion of M-cells to endothelioid cells varied with the age of the culture, after 2 days 3:1 and after 3 days 2:1 M-cells to endothelioid cell. In the same connection, Chacko (17), by cloning single heart-muscle cells from 5-day embryos, reported that, when cloned cells were attached, the contracting cells were round or spindle shaped. During the first 3 days in culture, these cells acquired one of two morphologies: approximately 40% of the cells became flattened or fibroblast-like, and about 60% were elongated. Furthermore, no significant difference was noted in frequency of beating between the F- and the elongated cells. Also a change in morphology was observed from elongated to flat and vice-versa: i.e. the F-cells transformed through intermediate shape to elongated cells. However, large numbers of cells that had neither the cardiac muscle cells morphology nor the ability to contract were observed in these (17) clonal cultures. Moreover, they were

fibroblastic and contained characteristic perinuclear granules in cytoplasm, but were distinguished as "non-muscle" cells because of their inability to contract, the absence of glycogen commonly observed in beating cells, and the lack of cytoplasmic fibrils. In addition, they were found (17) to proliferate very rapidly in low density cultures. The description of endothelioid cell's nucleus morphology presented above by Mark et al. (61) might, to some extent, resemble the nuclear description presented in paragraph 5 of Results. The nuclear morphology, as shown in this study, correlated with cellular activity in all cases. Thus mitotic activity, spread cells, and prolonged cultivation corresponded to an increase in nuclear volume and alterations in chromatin's shape and number of nucleoli. Nuclear alterations were more apparent and frequent in 12-day and 18-day than in 5-day cells. A matter of 10-15% un-identified morphological cell-types, (see later) observed in this study in all ages, cannot account for this general phenomenon. Moreover, similar correlations of cellular activity and nuclear alteration could be found in reports of Gurdon (29) and Harris (35, 36). Thus Gurdon (29), in nuclear transplantation experiments, observed that the transplanted nuclei undergo a rapid and pronounced increase in volume, and added that the degree of swelling of transplanted nucleus is always related to the rate at which it synthesizes DNA. Harris (35, 36) too, in cell hybridization experiments, reported that the morphological event associated with the reactivation of the nucleus is a progressive and massive increase in volume, and that this expansion of the nucleus is accompanied by dispersion of its chromatin. Furthermore, he indicated that the nuclear enlargement is directly related to the amount of RNA synthesized by the nucleus.

From the results obtained and the observations by the various authors already referred to in this chapter, it is apparent that the morphological classification of heart cells in culture as M-cells and F-cells is doubtful. Obviously, the genotypic permanency of these cells was, in proper culture conditions, manifested by demonstrating two characteristic properties of heart cells: 1) functional differentiation, and 2) structural differentiation. However, cultures with a totally differentiated cell population were not observed in this study. Maximum values observed did not exceed 86%. This discrepancy might be the consequence of two factors: 1) either the culture conditions were not sufficient to permit all the cells to differentiate in a given culture, 2) or presence of the "endothelioid" or "non-muscle" cells

observed by Mark et al. (61) and Chacko (17) might have contributed to establish a heterogenous cell population of muscle and non-muscle cells. The latter presumption has actual significance, as, in this study, the appearance of a certain percentage of striated cells was not a constant phenomenon even in experiments performed under similar conditions. In order to clarify this problem, 2 series of experiments were performed in which cells from 18-day embryos were isolated and cultured in low cell densities (~ 100 cells/mm²) and fixed and stained after 24 h, the proportion of morphologically cell types being calculated. The average values obtained are roughly: 20% round cells, 23% F-cells, 46% M-cells and 11% unidentified cells (Plate 6). Comparable values for 5-day cells can be seen in Plate 5. As because the round cells are unstretched myoblasts (17) (Plate 22), and F- and M-cells are derived from the same myogenic origin (Plates 23, 24, 25), as earlier discussed, the proportional contribution of non-muscle cells to the total population, is not more than 10-15% in any age. Therefore, the different growth capacities presented in Chapter two cannot be the result of the overgrowth of non-muscle cells, because the non-muscle cells reported by Chacko (17) were found in clones of 5-day cells, and in this study, the growth capacity of these cells was relatively very low. Thus, obviously variations in phenotypic expression (Fig. 2) result from uncontrolled variations in the culture conditions. The experiments described in Chapter one showed that different sera had varied effects on expression of differentiation. Furthermore, it must be taken into account that 30% serum in 70% Tyrode's BSS might not be a complete and efficient medium to allow a very high degree of differentiation. None the less, in the course of this experimental period, improvements in culture conditions brought about improvements in phenotypic-expression. For instance, it was found that supplementation of 2% EE or addition of 16 mg/ml bovine-albumin or substitution of Tyrode's BSS by M199 or Eagle's (MEM) media considerably improved the differentiation, both in the single cell and in a cell population. Although structurally differentiated single cells were occasionally observed (Plates 22-27), the increase in initial cell densities to optimum levels (400-500 cells/mm²) remarkably resulted in a higher proportion of differentiated cells. A similar effect was noted when the beating was studied.

Growth-medium



Basal medium

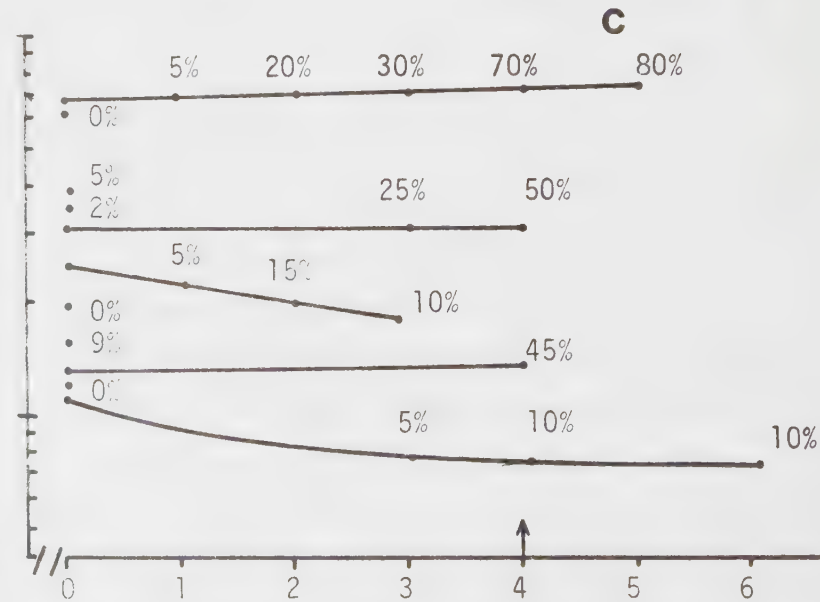
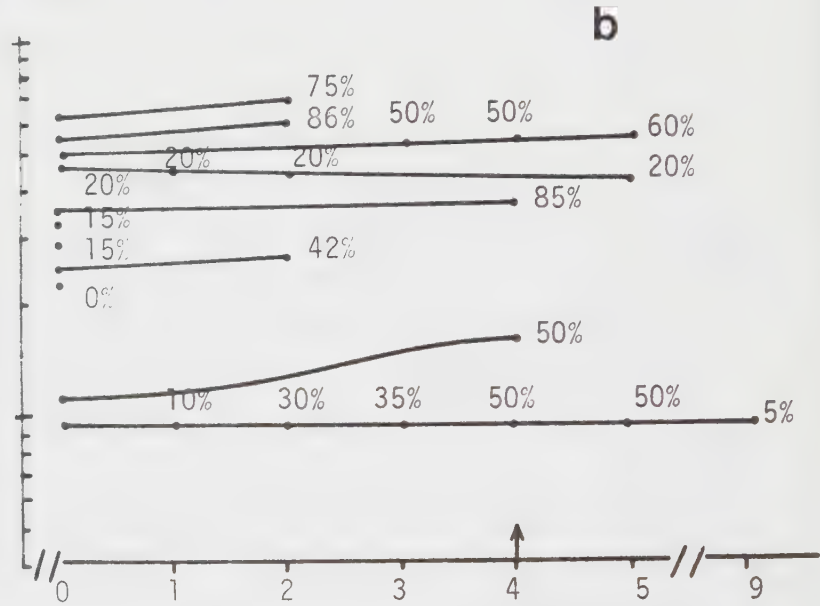
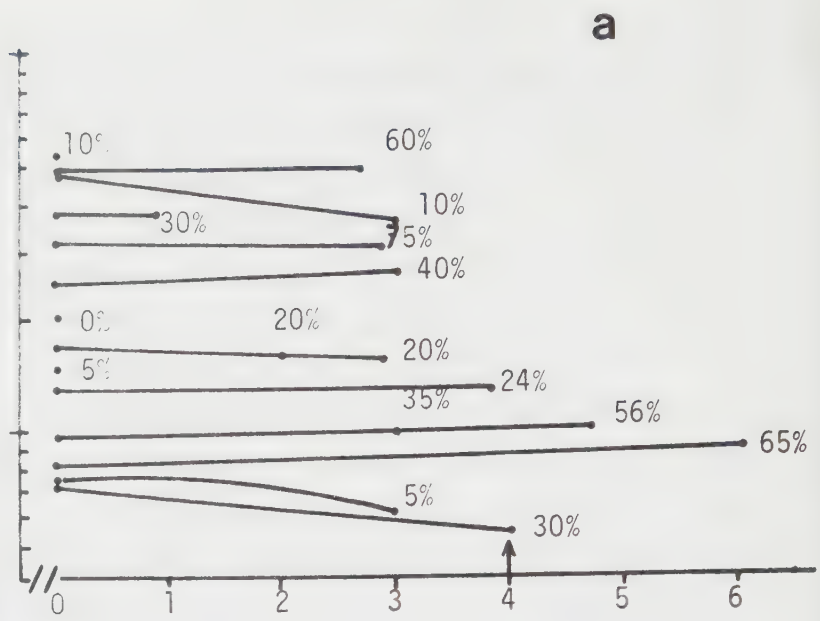


Fig. 1. Growth and differentiation of 5-, 12-, and 18-day cells in GM.

Fig. 2. Growth and differentiation of 5-, 12-, and 18-day cells in BM.

In Fig. 1, 2, and 3, the abscissa represents days and the ordinate cells/dish. The g.r. refers to the exponential growth rates of the different curves from top to bottom, respectively. The % values give the percentage of differentiated cells (% d.c.)/dish.

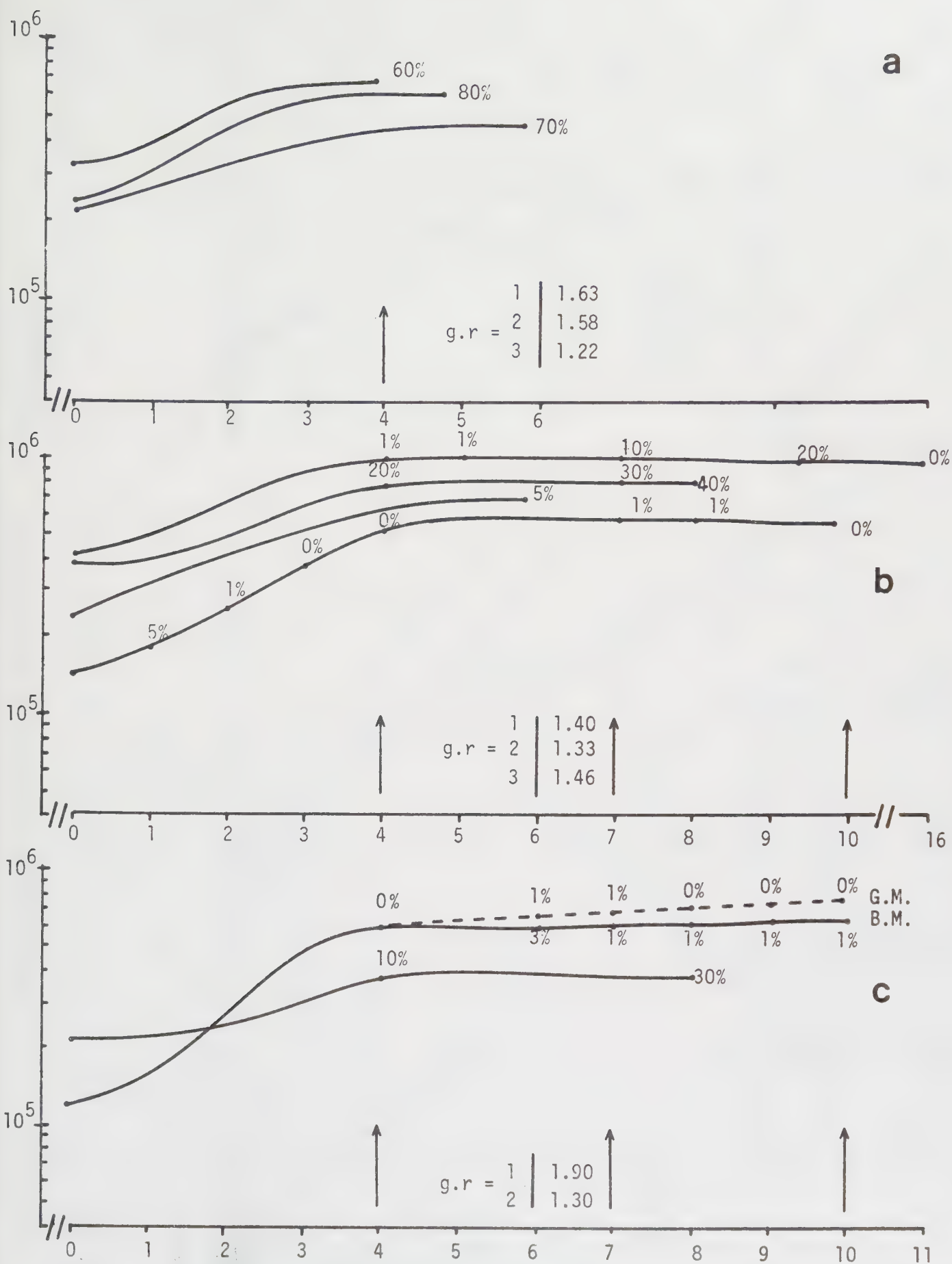


Fig. 3. Growth and re-differentiation of 5-, 12-, and 18-day cells in growth and basal medium. The solid line $\circ-\circ$ indicates the replacement of GM by BM; the broken line $\circ---\circ$ indicates the replacement of GM by new GM. The arrows indicate the renewal of these media.

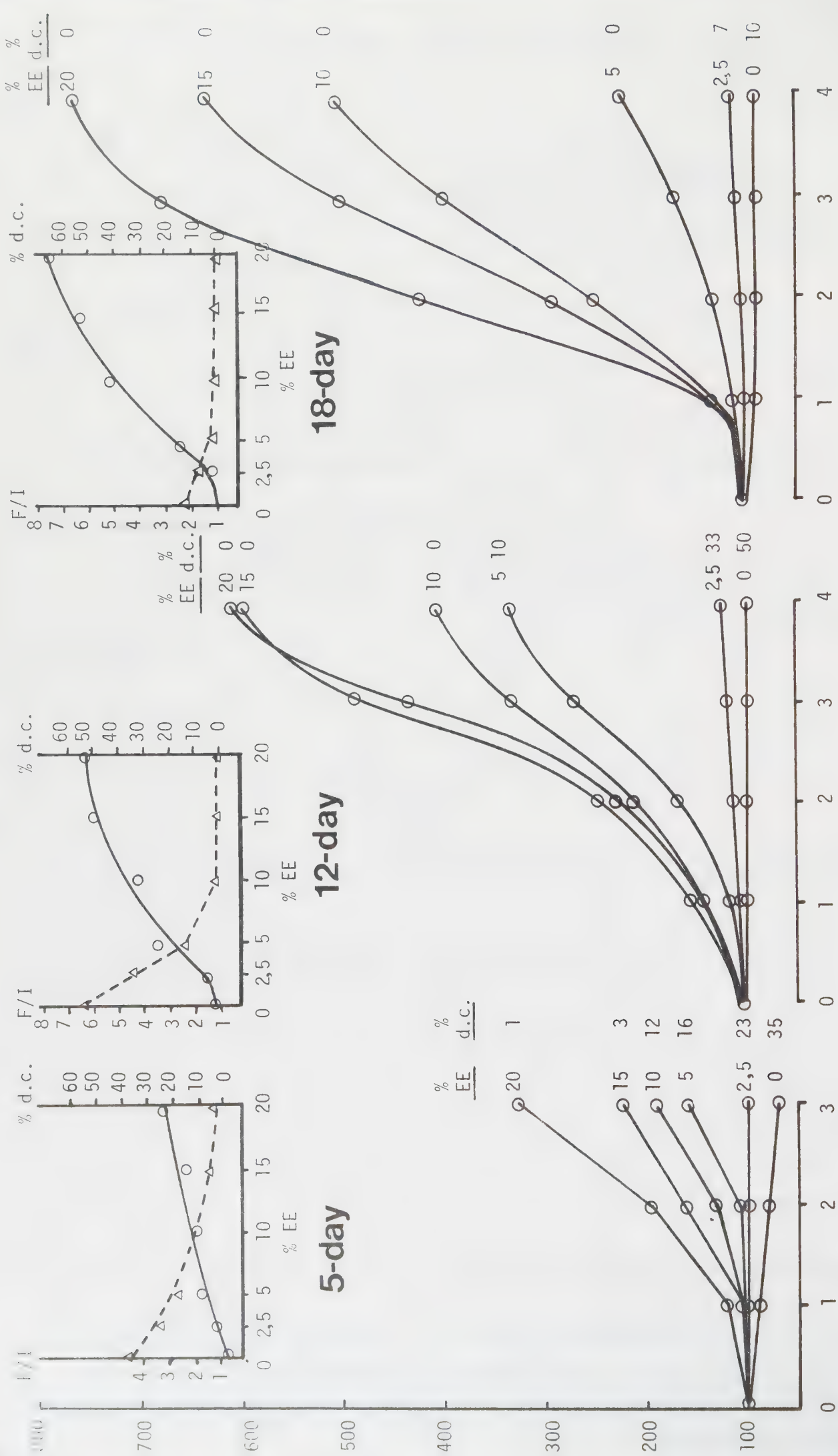
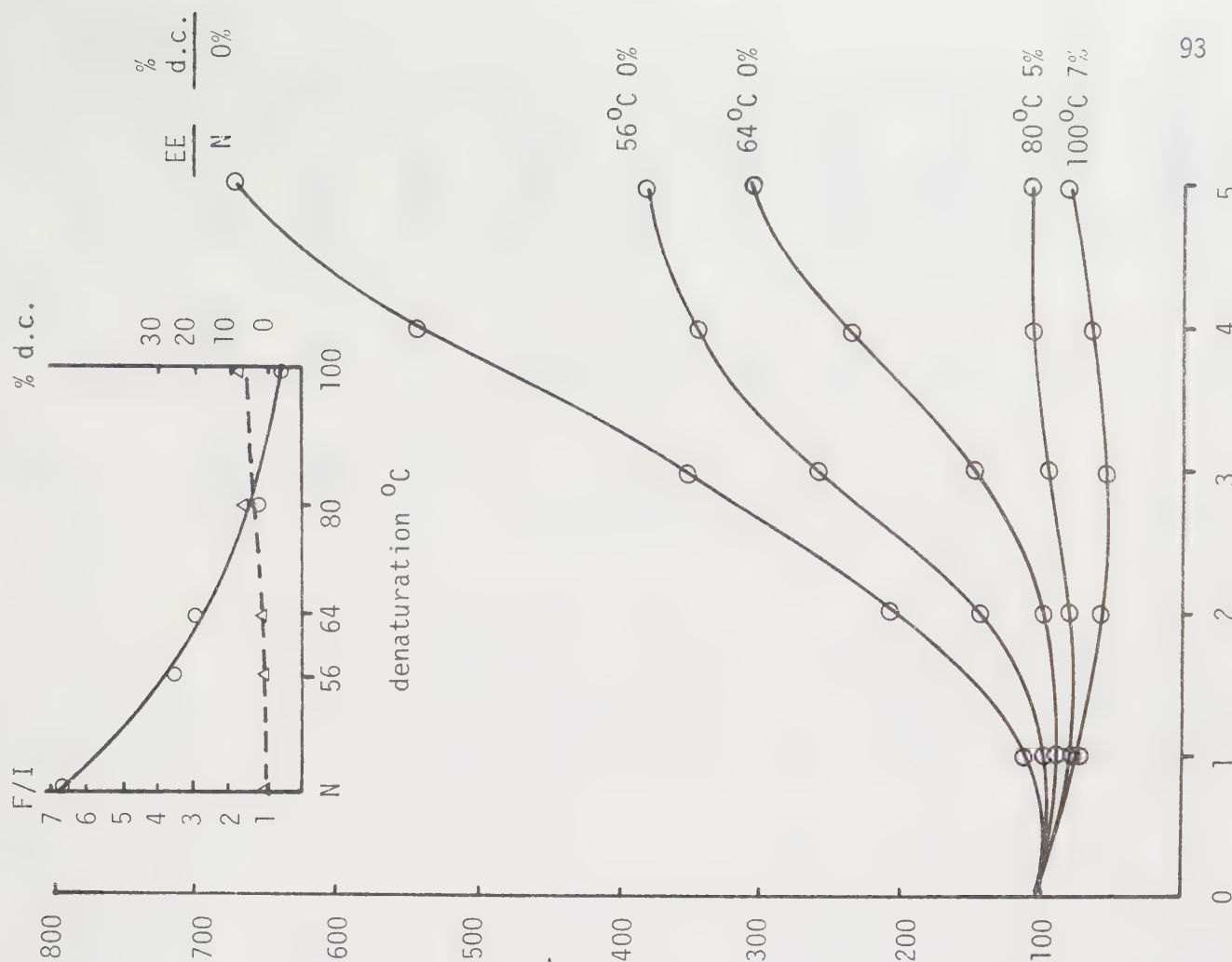
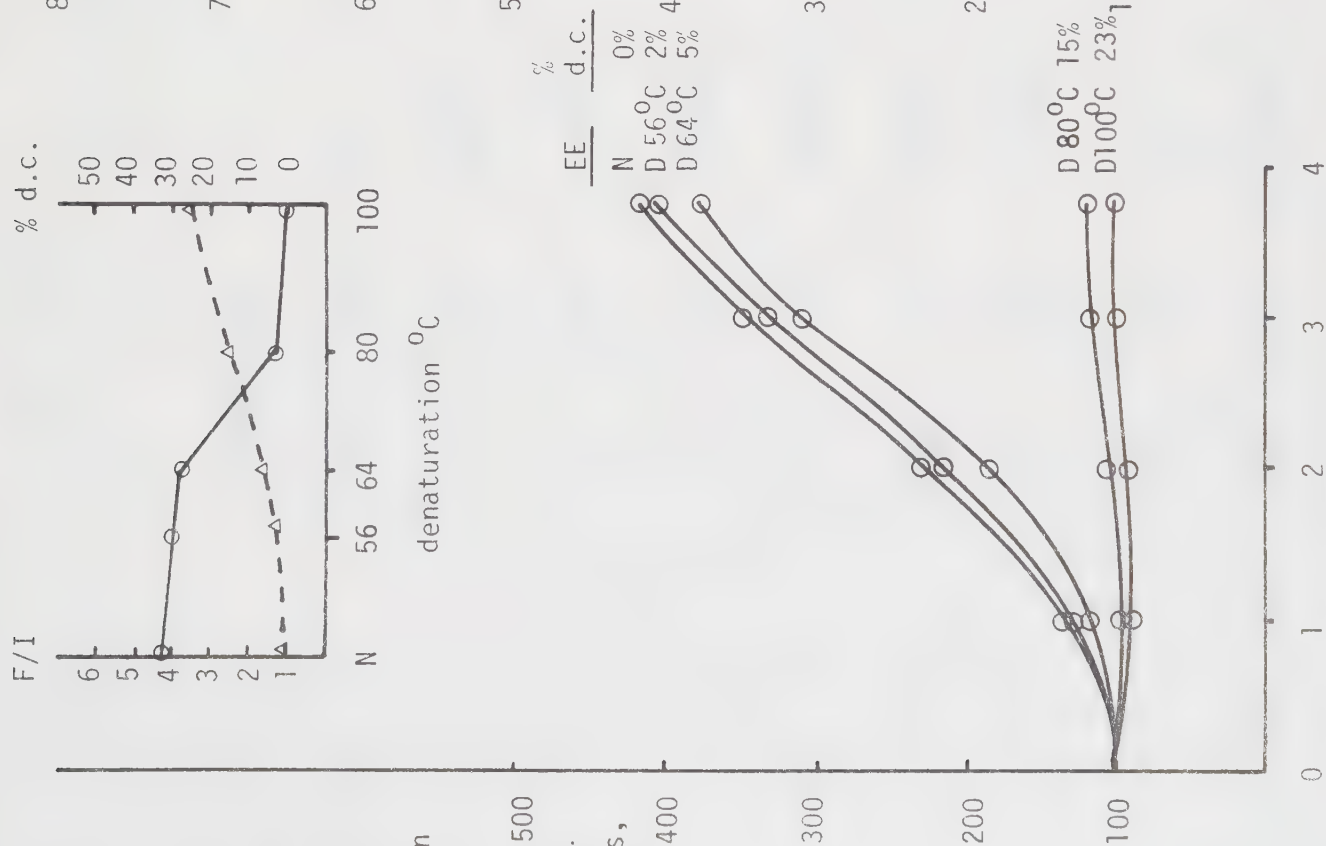
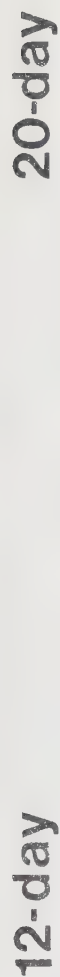


Fig. 4. Abscissa: days, ordinate: x 1000 cells/dish. Growth and differentiation of 5-, 12-, and 18-day cells in 20% DH.S. supplemented with different concentrations of EE. F/I, O—O indicates the ratio of final cell number/initial cell number. Δ — Δ indicates the % of differentiated cells/dish.

Fig. 5
Growth and
differentiation
of 5-, 12-, and
18-day cells in
normal (N) and in
denatured EE in
different
temperatures (D).
For other symbols,
see Fig. 4.



cells/dish

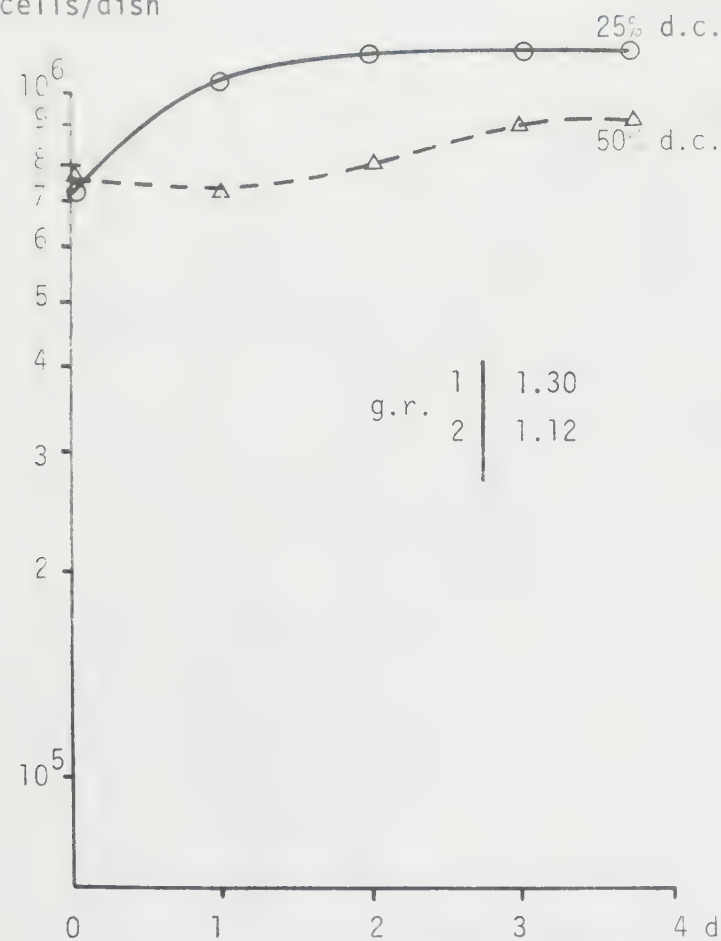


Fig. 6. Growth and differentiation of 7-day cells in GM $\circ-\circ$, and in BM $\triangle--\triangle$.

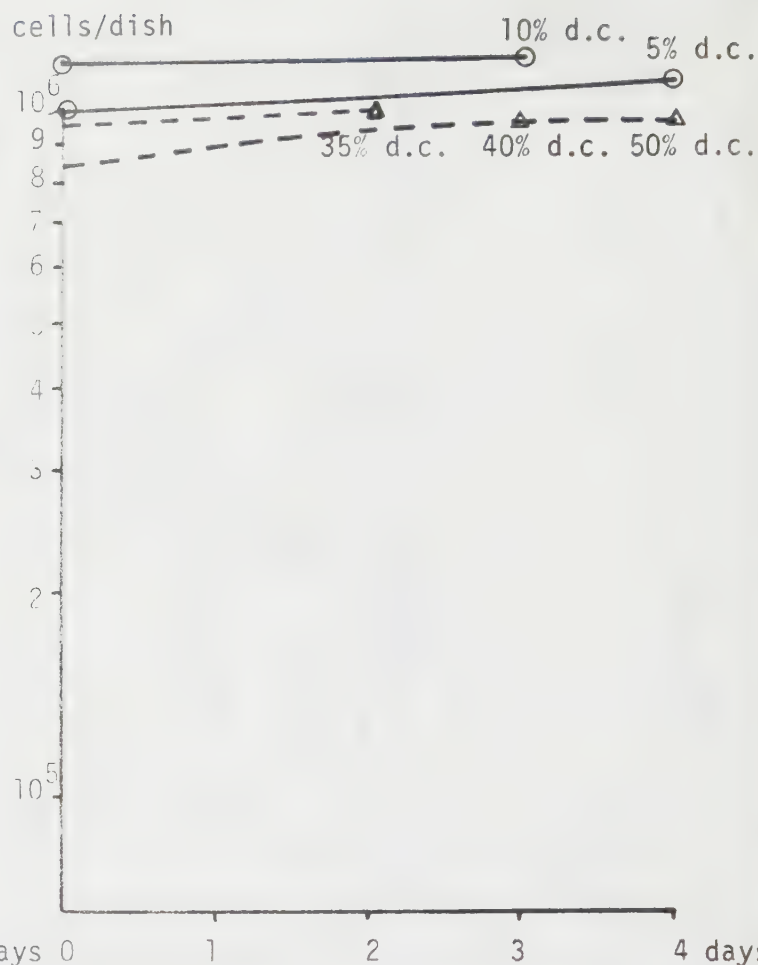


Fig. 7. Growth and differentiation of 12-day cells in GM $\circ-\circ$, and in BM, supplemented with 2% EE $\triangle--\triangle$.

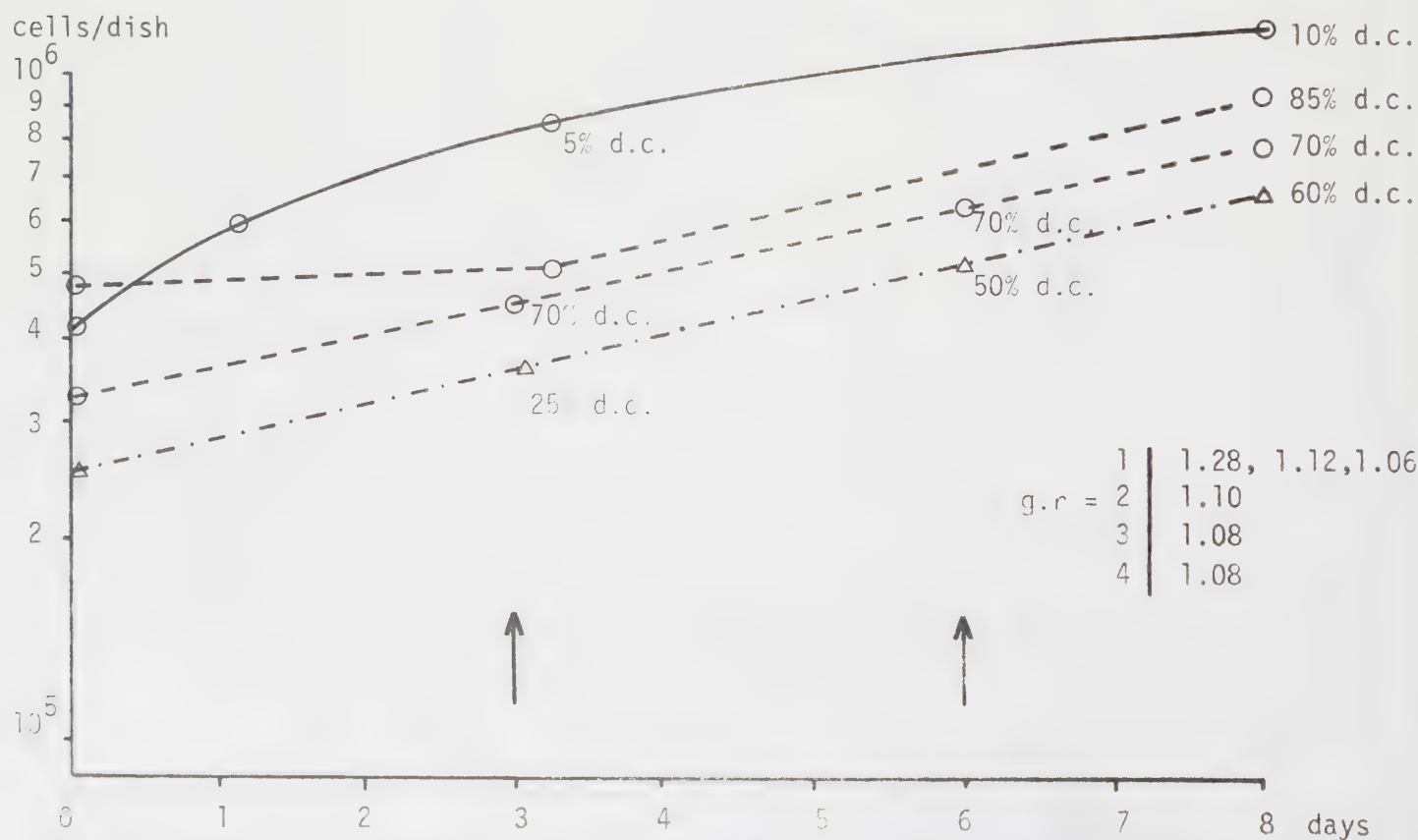


Fig. 8. Growth and differentiation of 12-day cells in GM containing different concentrations of EE for a period of 3 days, and consequently in basal medium for an additional 5 days. Arrows indicate the replacement of GM to BM. $\circ-\circ$ 20% DH.S. + 10% EE. $\triangle--\triangle$ 25% DH.S. + 5% EE and $\circ--\circ$ 27.5 DH.S. + 2.5% EE.

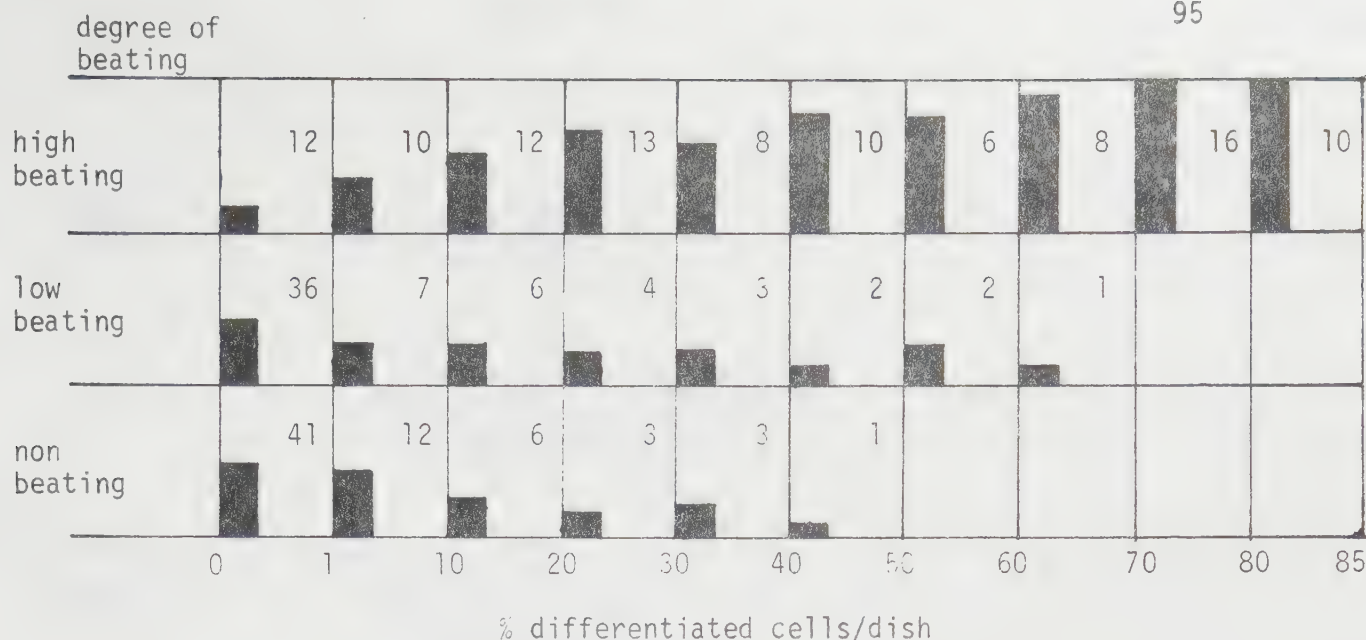


Fig. 9. The correlation between the degree of beating¹⁾ and the % of differentiated cells/dish. The degree of beating was evaluated before fixation and staining. The numerical values indicate the number of observations from a total of 232 dishes when a certain degree of beating was observed in a certain % of d.c./dish. The black columns indicate the proportion of the observations.

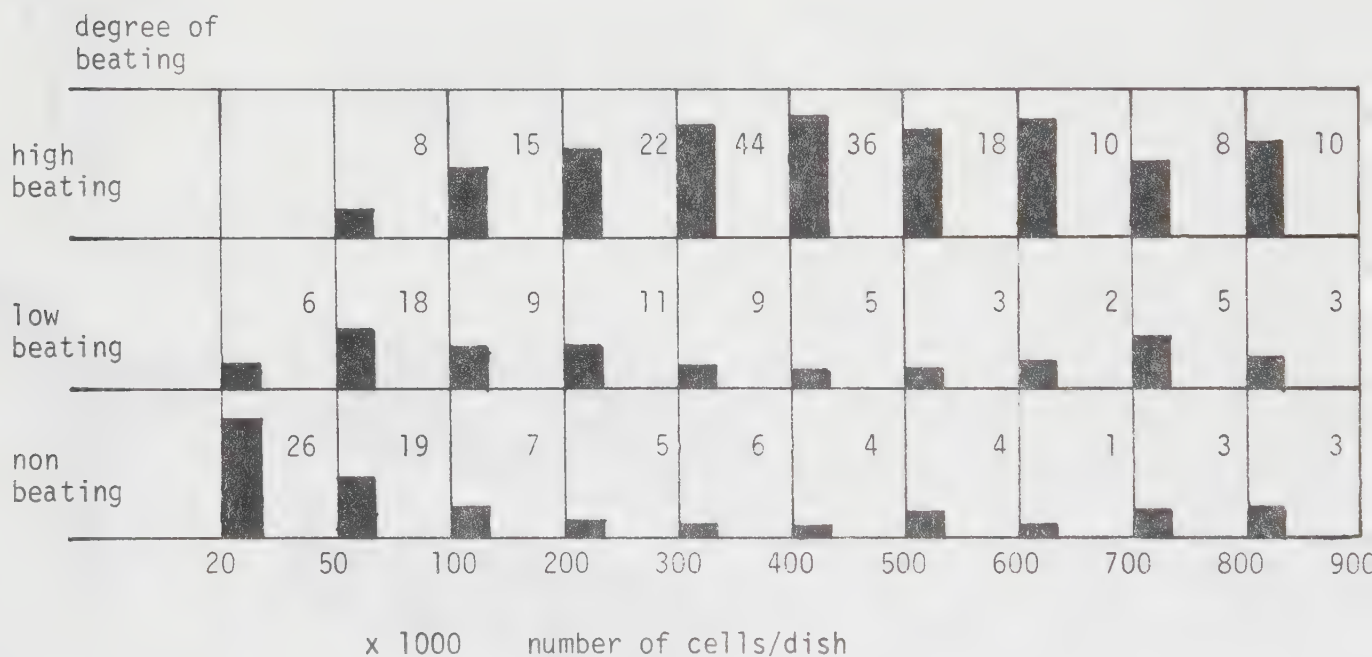


Fig. 10. The correlation between the degree of beating¹⁾ and cell concentration (cells/dish). The degree of beating was evaluated at the time of cell counting during the culture period. The numerical values indicate the number of observations out of a total of 320 that exhibited a certain degree of beating within the defined number of cells/dish. The black columns indicate the proportion of the observations.

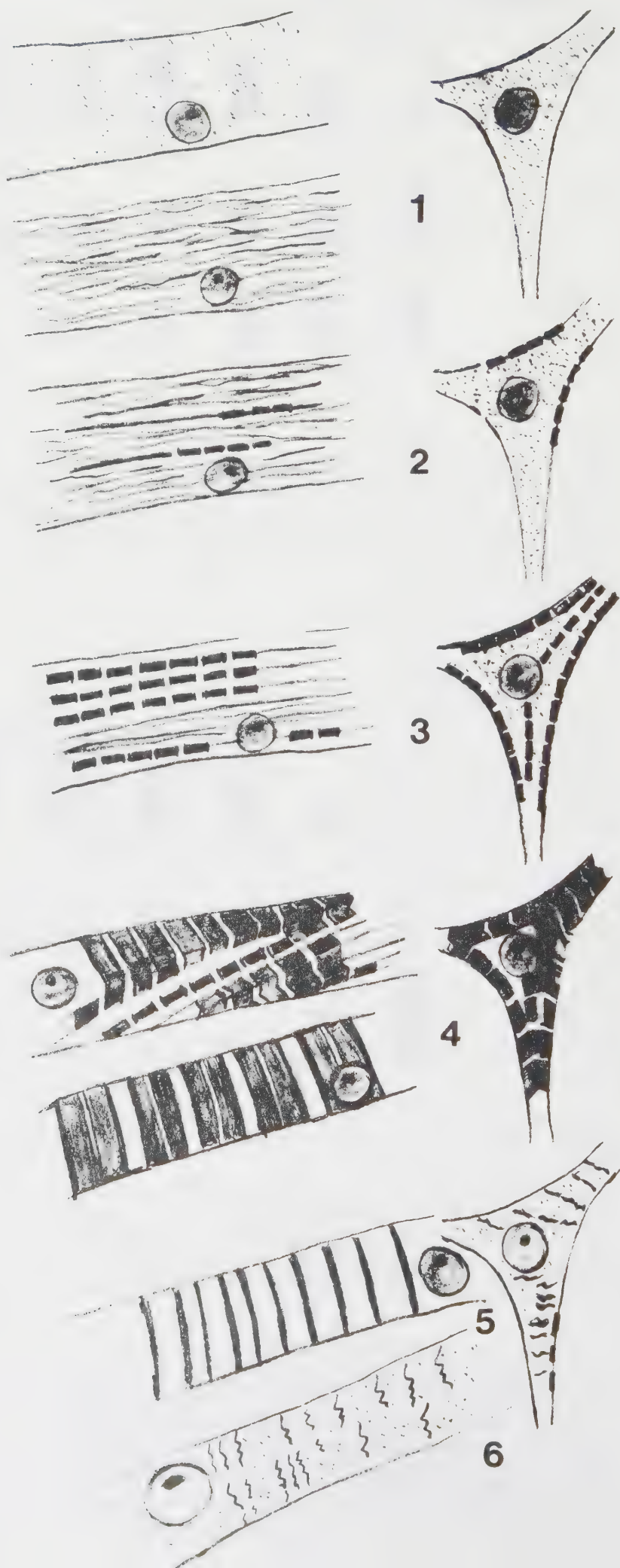
1) Arbitrary units employed to evaluate the spontaneous contraction of the cultures immediately after they were taken from the incubator, under phase contrast observations in a magnification of x 100. The cultures were classified in 3 groups: a) non-beating, in which no spontaneous contraction was observed; b) low-beating, in which relatively few cells demonstrated spontaneous contraction; c) high-beating, in which considerable numbers of cells demonstrated spontaneous contraction.

M-cell

F-cell

Fig. 11.

Schematic drawing of evolution and deterioration of the cross-striation in culture. No marked morphological alterations in nucleus could be observed during this procedure up to stage 6 from then on, the nuclear volume seems to increase.



PLATES

Plates 1-4. Electronmicrographs of intact and trypsinized hearts of 5-day and 18-day old chick embryos.

Hearts from 5- and 18-day old embryos were isolated and the ventricular ends were cut into small pieces. After 3 washes in Tyrode's BSS, the heart pieces of each age were divided into two parts. One part was fixed directly, and the other part was trypsinized in the usual way (see material and methods, Chapter 2). The obtained cell suspension was centrifuged at 1000 rev/min for 5 min in a conical test tube. The pellet and the intact (not trypsinized) pieces from each age were fixed in 2.5% glutaraldehyde in millonig buffer for 2 h, and after washing in the buffer solution, were fixed in 1% OsO_4 for 2 h. Thereafter, the pieces and the pellets were embedded in Vestopal, and ultrathin sections were cut on an LKB ultratome and inspected in a Philips 300 electronmicroscope.

Plate 1. Electronmicrograph of intact ventricular end of 5-day embryonic heart. Magnification: $\times 18,800$.

Plate 2. Electronmicrograph of intact ventricular end of 18-day embryonic heart. Magnification: $\times 18,800$.

Note that, unlike the 5-day heart, the cross-striated myofibres are much more abundant and occupy almost all the cytoplasmic space in the 18-day heart.

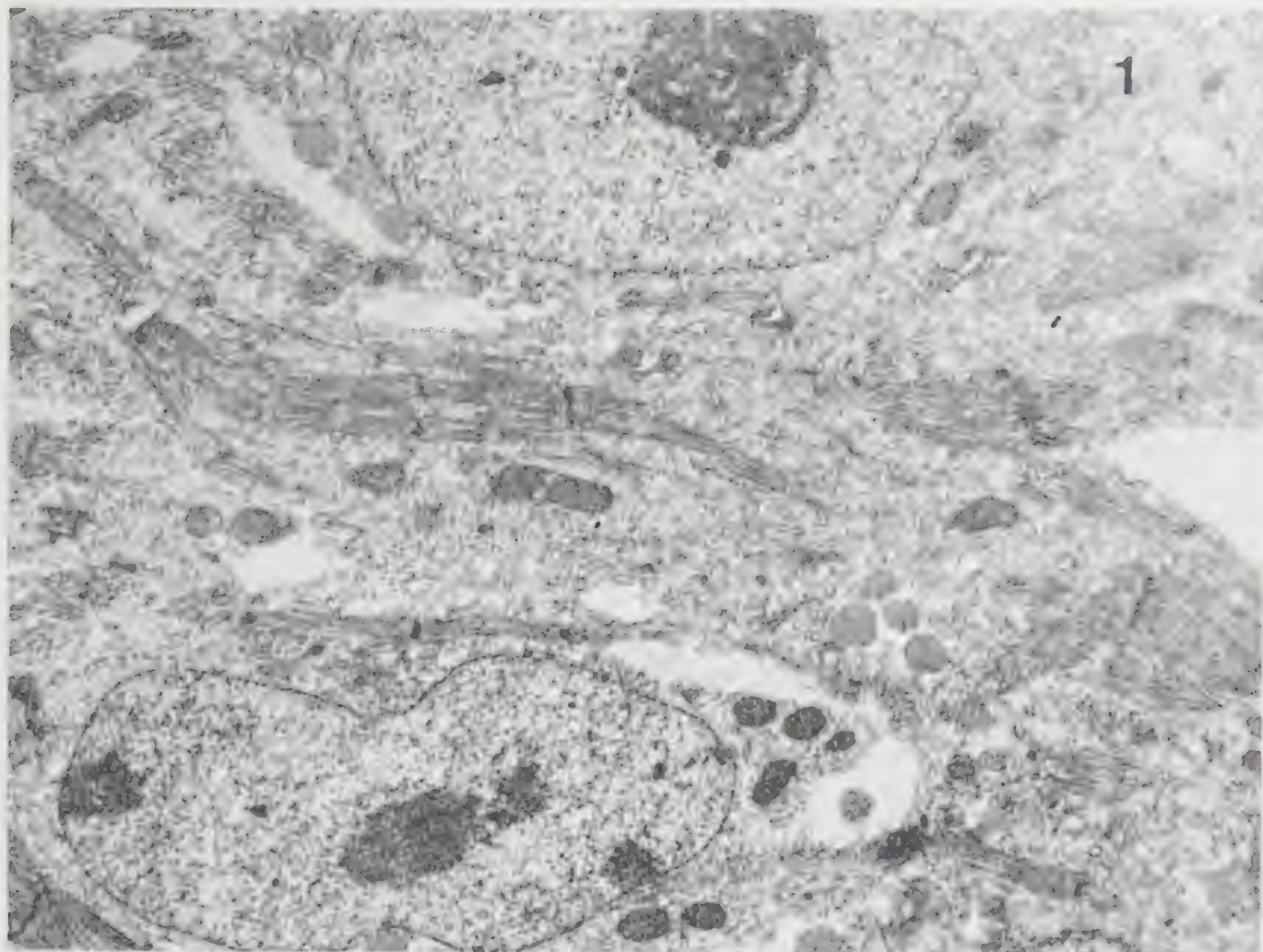


Plate 3. Electronmicrograph of trypsinized ventricular end of 5-day embryonic heart. Magnification: x 8,800.

Plate 4. Electronmicrograph of trypsinized ventricular end of 18-day embryonic heart. Magnification: x 10,600.

Note: the action of disintegration causes most of the cells to lose their original differentiation. However, occasional cells after trypsinization still reveal some degree of cross-striation (arrows).

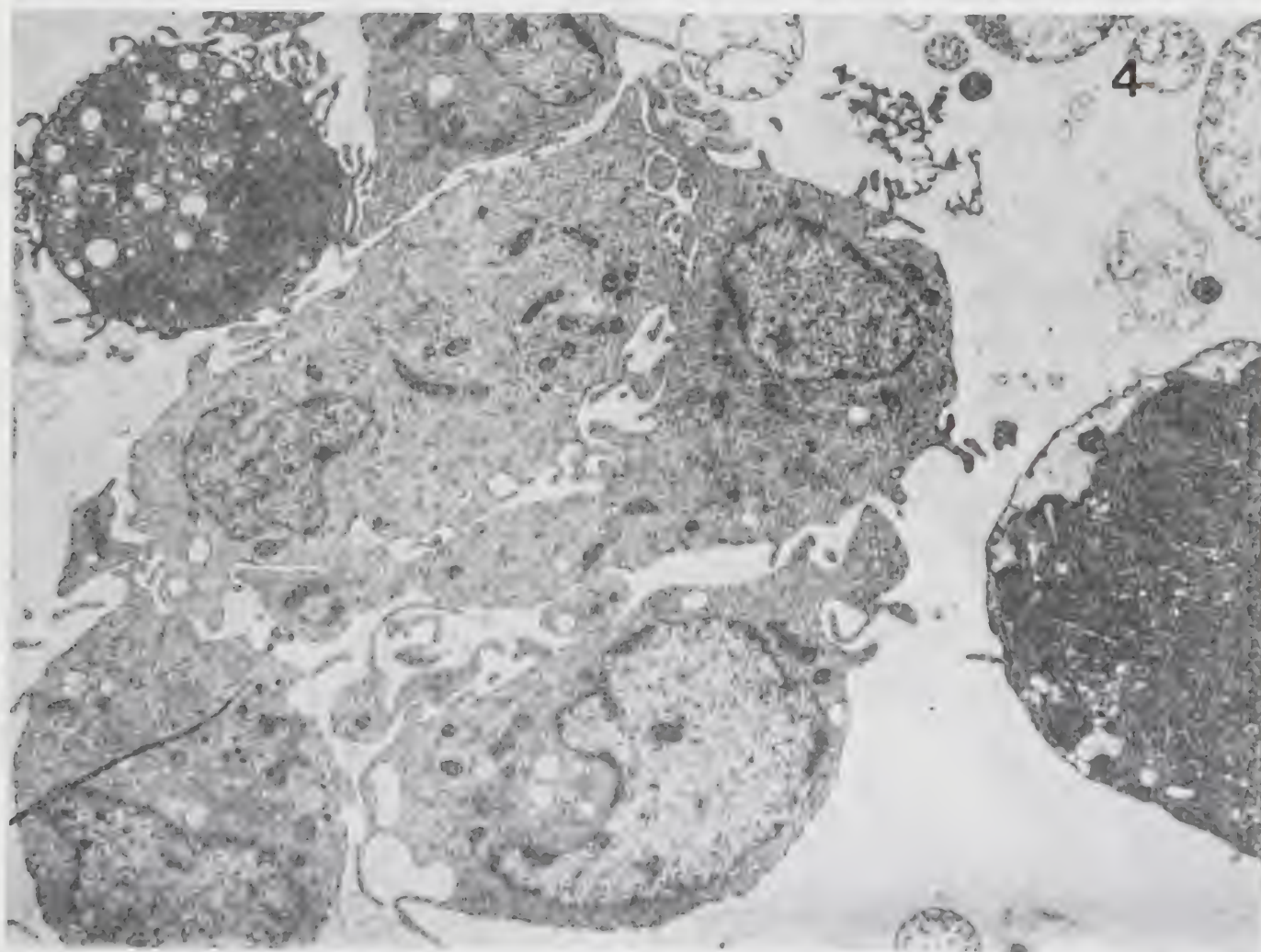
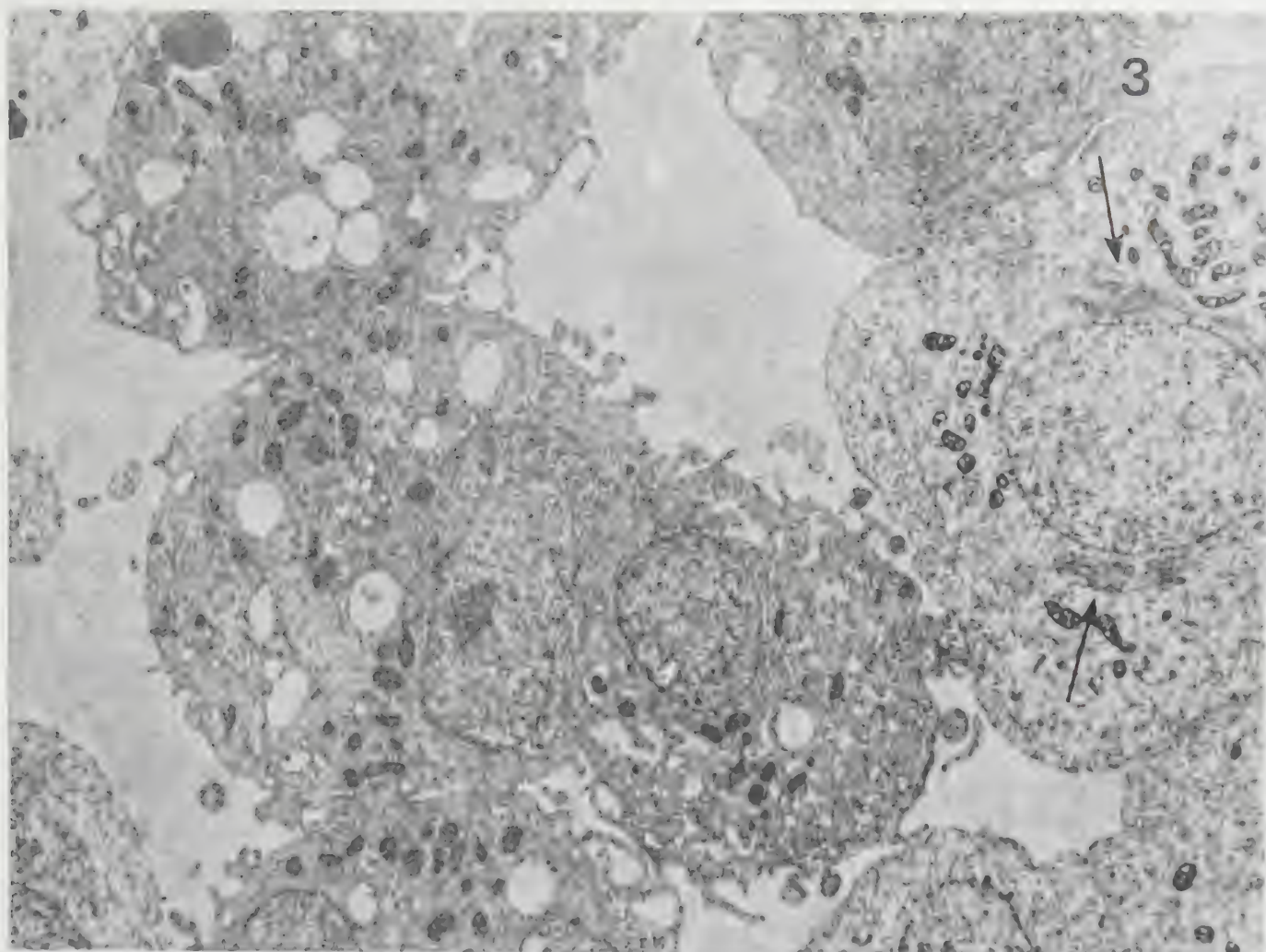


Plate 5. Morphological appearance of disintegrated 5-day embryonic heart cells 20 h after plating in BM. c.d. = 4×10^5 cells/dish. - x 160.

Plate 6. Morphological appearance of disintegrated 18-day embryonic heart cells 40 h after plating in BM. c.d. = 5×10^5 cells/dish. - x 160.

Plate 7. 13-day heart cells, 3 days in culture. The cells were grown in BM (20% D Calf. S + 80% Tyrode's BSS) supplemented with 16 mg/ml bovine-albumin. c.d. = 5×10^5 cells/dish. In these conditions, more than 80% of the cells were completely cross-striated. - x 400.

Plate 8. 13-day heart cells, 3 days in culture in BM (20% D Calf. S + 5% EE + 75% Eagle (BM) medium). c.d. = 4×10^5 cells/dish - more than 80% of the cells are myofibrillated but without cross-striations. - x 1,000.

Plate 9. 13-day heart cells, 3 + 1 days in culture: 3 days in BM (as in Plate 8) + 1 day in BM (20% D Calf. S + 80% Tyrode's BSS). Few fibres are partly cross-striated. x 1,000.

Plate 10. 13-day heart cells, 3 + 2 days in culture: 3 days in GM (as in Plate 8) + 2 days in BM (as in Plate 9). The cross-striation develops longitudinally in single fibres. - x 1,000.

Plate 11. 13-day heart cells, 3 + 5 days in culture: 3 days in GM (as in Plate 8) + 5 days in BM (as in Plate 9). Many fibres developed cross-striation. The closed differentiated fibres are associated and laterally give rise to large cross-striated sheets. - x 1,000.

Plate 12. The appearance of the decomposing cross-striations. This feature can appear throughout of the culture, but is more frequent in older ones. 12-day heart cells grown for 5 days in BM. Only the A-bands are present. - x 1,000.

Plate 13. The appearance of the advanced decomposed cross-striations. The A-bands are suffering further dissolution. 12-day heart cells in BM, 6 days in culture. - x 1,000.

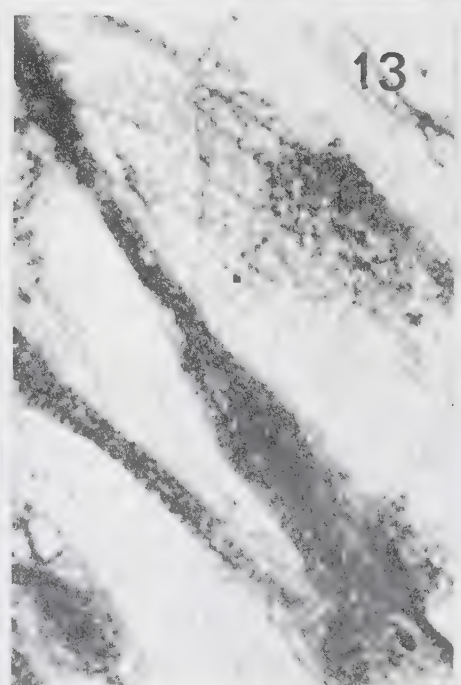
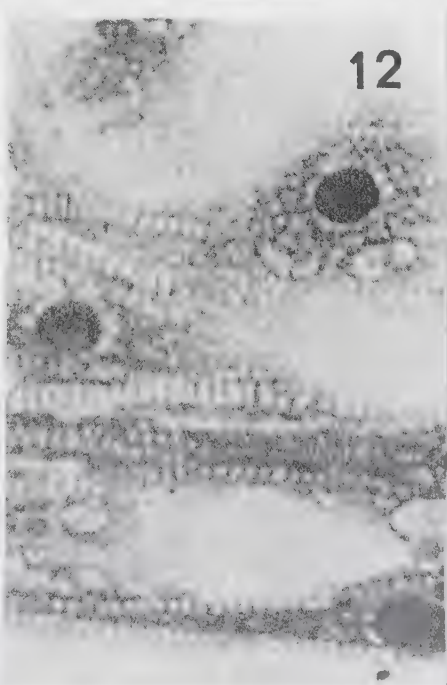
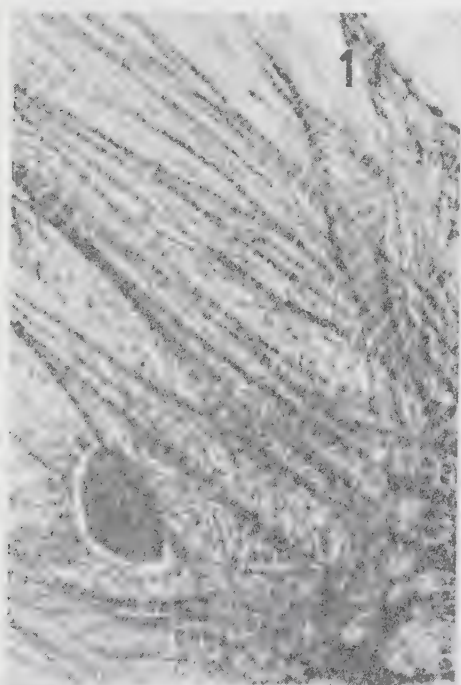
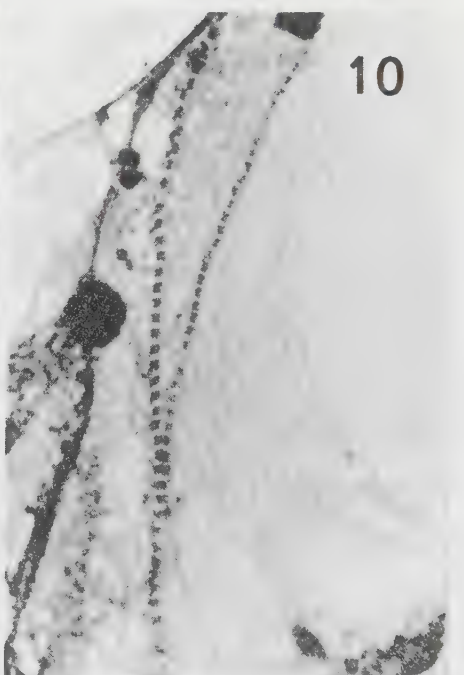
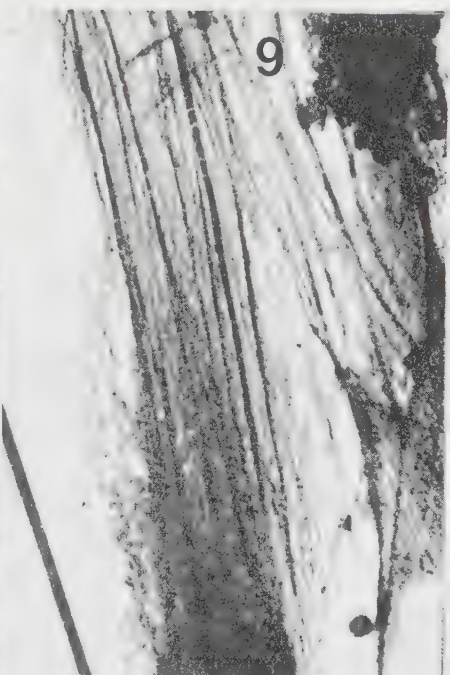
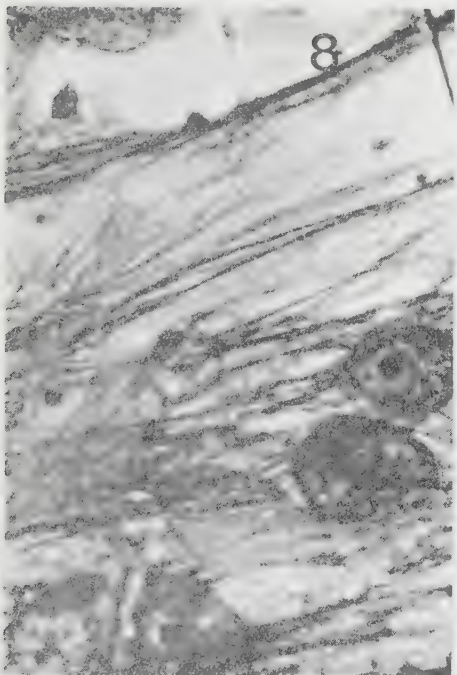
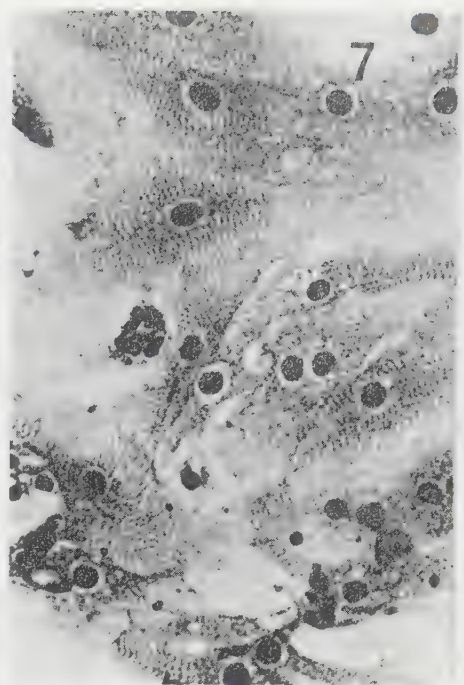
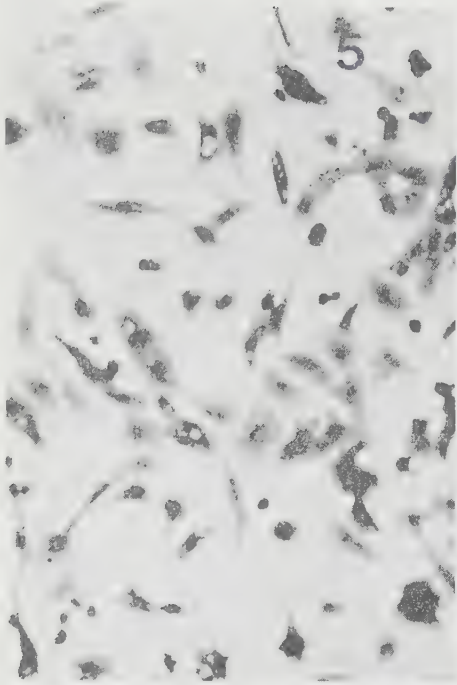


Plate 14. 12-day heart cells grown in high concentration of EE (20% DH.S. + 20% EE + 60% Tyrode's BSS) 4 days in culture. Cytoplasmic disorder. Relatively large and active nuclei with more than one nucleoli characterize the heart cells in these conditions. c.d. = 2×10^5 cells/dish. - x 400.

Plate 15. The above picture in higher magnification. The destruction of the cytoplasmic structure is easily seen. - x 1,000.

Plate 16. 12-day heart cells, cultivated 4 days in GM + 4 days in BM. Initial cell density = 2×10^5 cells/dish; final cell density, 8×10^5 cells/dish. The cells experienced an active proliferation phase and a long cultivation period. Cells react to such conditions by loss of differentiation, marked increase in nuclear volume, and chromatin activity. - x 1,000.

Plate 17. 18-day heart cells, 3 days in culture in GM. c.d. = 4×10^5 cells/dish. The above symptoms (see plate 16) appear more readily in 18-day cells grown in GM. - x 720.

Plate 18. 18-day heart cells, 2 days in culture in GM. c.d. = 2×10^5 cells/dish. The mentioned symptoms (see plate 16) are further stressed in spread cells. - x 720.

Plate 19. 18-day heart cells, 6 days in culture in GM. c.d. = 4×10^5 cells/dish. The mentioned symptoms (see plate 16) are intensified by continued cultivation. The appearance of fat granules can be observed. - x 720.

Plate 20. 12-day heart cells, 16 days in culture (4 days in GM + 12 days in BM). Note the total occupation of cytoplasmic space by fat granules. - x 720.

Plate 21-28. Morphological appearance of differentiated cell types in culture.

Plate 21. 12-day heart cells, 3 days in culture in GM + 2% EE. c.d. = 4×10^4 cells/dish. Various differentiated cell types, e.g., M- and F-cells can be observed. - x 290.

Plate 22. An isolated 18-day differentiated round cell, 40 h culture in BM. -x 1,000.

Plate 23. An isolated 5-day differentiated M-cell, 2 days culture in BM. -x 1,000.

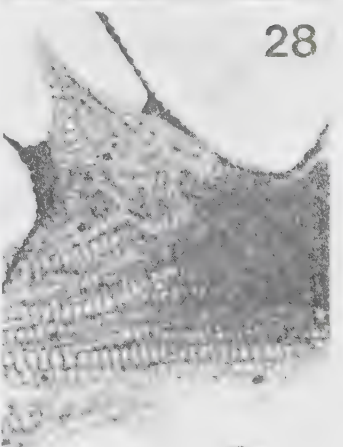
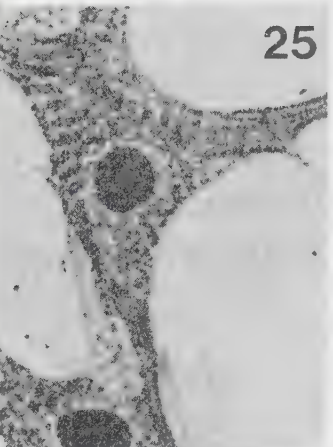
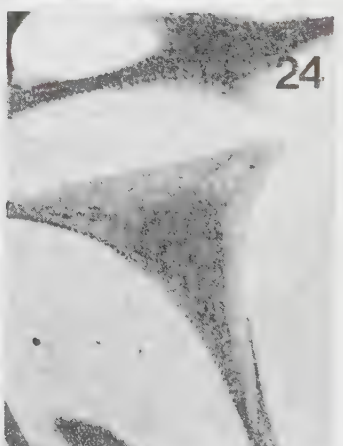
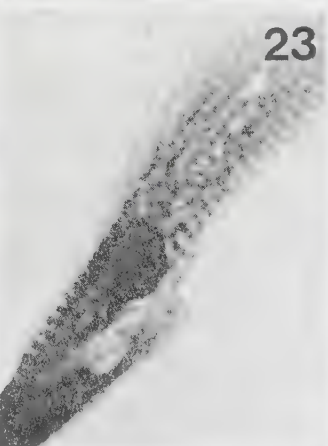
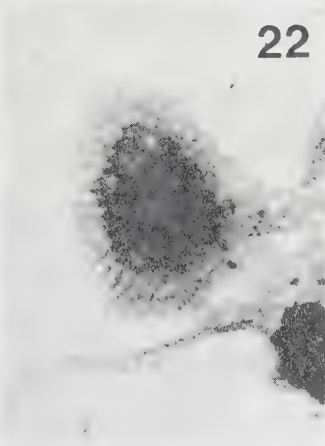
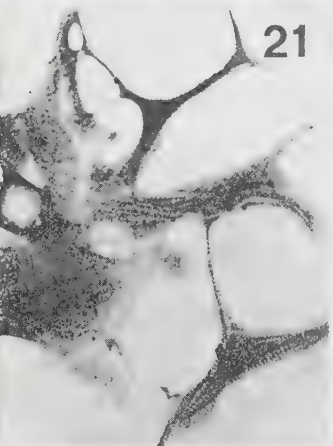
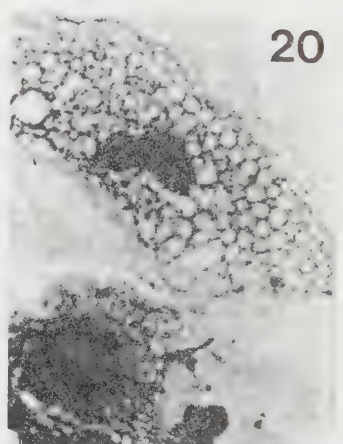
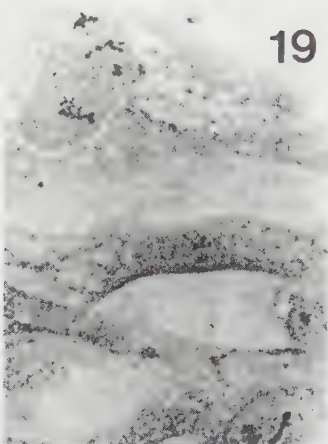
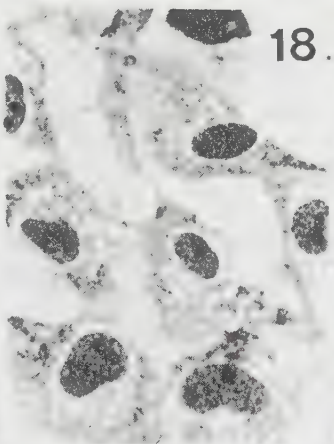
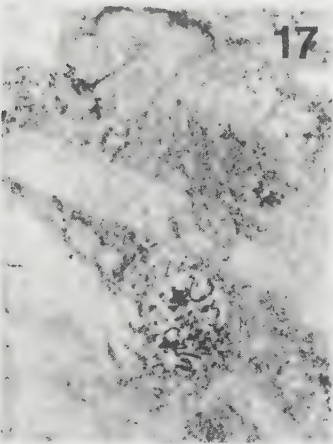
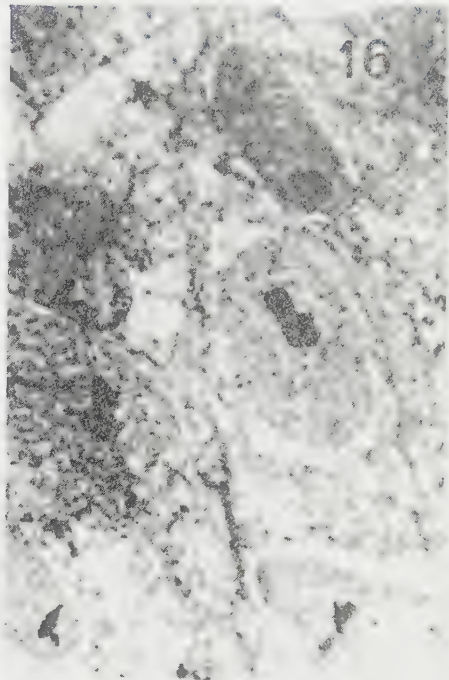
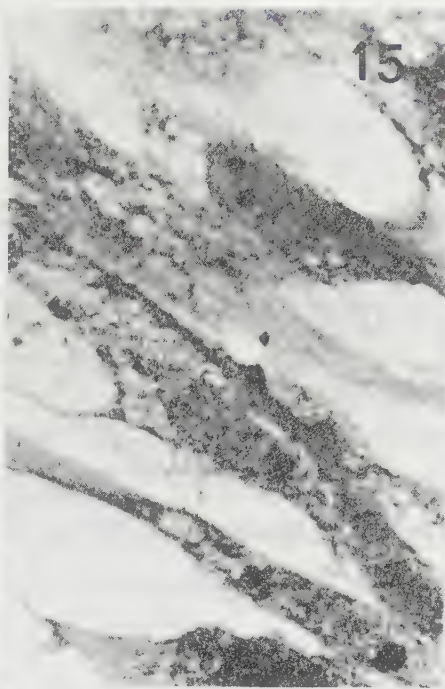
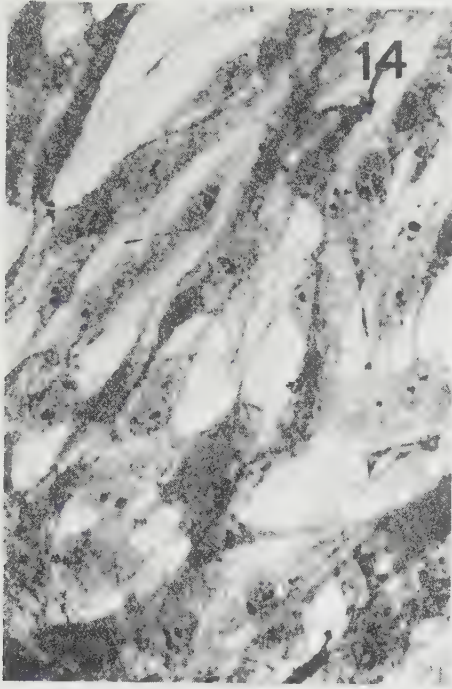
Plate 24. Two isolated 13-day differentiated F- and M-cells, 3 days culture in BM. - x 400.

Plate 25. An isolated 18-day differentiated F-cell, 4 days culture in BM. - x 1,000.

Plate 26. An isolated 5-day differentiated triangular cell, 3 days culture in BM - x 720.

Plate 27. An isolated 12-day differentiated rectangular cell, 3 days culture in BM + 2% EE. - x 720.

Plate 28. Two differentiated irregular shaped 12-day cells, 3 days culture in BM + 2% EE. - x 720.



GENERAL SUMMARY

1. A wide range of sera from large variations in origin, age of donor, and physical treatments were incorporated in culture media, and their effects on survival and growth of three different types of cells¹⁾ were studied. Simultaneously, an electrophoretic analysis of the sera was performed.
2. Although no rules were established concerning the behaviour of cells in different types of sera, some remarkable tendencies in behaviour of cells in a common serum were noted. The normal (not dialysed) sera were classified in three groups according to their effects: a) toxic sera in which the cells died in a relatively short time; b) indifferent sera in which the cells survived, but neither grew nor declined; and c) promoting sera in which the cells survived and proliferated. In addition, the cells in the toxic sera experienced two major symptoms: shrinkage and fattening. Significant differences were found in the ability of the normal sera to support attachment, stretching, growth, beating, and differentiation of the various cells.
3. Dialysis moderated the action of both toxic and promoting sera: primarily, both toxic and growth promoting factors were present in dialysate fraction. The established cell-lines could proliferate even in the presence of a simple BSS supplemented with dialysed macromolecular fraction of some serum samples. Dialysis apparently does not remove all the LMW substances associated with toxicity, promoting, and functional activity.
4. No correlation between behaviour of cells and the macromolecular lipoprotein fraction could be found in these culture conditions.
5. A high level of albumin in serum was shown to exert toxic effects on cells in culture. A serum was toxic when the albumin fraction amounted to more than 40% of the total protein, or when the absolute quantity exceeded a certain level. But the toxicity did not appear gradually: it was a threshold effect. However, the toxicity of albumin fraction was not caused by the "albumin protein" per se, but by the substances associated with it. The biological function of albumin as a carrier in lipid metabolism was further emphasized. None the less, the globulin fraction alpha + beta + gamma

1) primary chick embryo heart cells; also established Chinese hamster cells and Ehrlich ascites tumour cells.

($\alpha+\beta+\gamma$), even in minute amounts, mainly determined the promoting quantity of the serum. Moreover, sera with a relatively high total protein content showed toxic effects if the relative contribution of the albumin fraction exceeded a certain level. Toxicity was associated also with a serum sample relatively rich in distinct proteins (as observed in electrophoretic pattern by additional bands).

6. Heart cells grown in pooled sera from several individuals became more susceptible to the culture conditions. In general, growth of heart cells was more stabilized when, before application to the culture medium, the serum was heat denatured (56°C for 30 min). Moreover, the susceptibility of heart cells in pooled sera decreases too. Increasing the denaturation temperature (64°C for 12 min) increases the growth stimulatory activity and the stabilizing property of the sera.

7. Heart tissue from chick embryos of different ages were proteolytically (trypsin) disintegrated. The resulting cells were cultivated in a permanent growth medium, and the growth pattern was followed until the stationary phase was reached.

8. In this closed system, the initial cell concentration per unit of area (1 mm^2) was found to affect the final cell density. In relatively high or low cell inocula, the proliferation ceased. The limits of initial cell densities necessary for growth were smaller for cells from younger embryos (4-5 days) than from older (18-19 days). The dependence on inoculum size was quantitatively different for cultivated myoblasts of different embryonic age. Within an intermediate range of cell concentration which allowed optimum growth, the final cell count per initial cell count (F/I) was much greater for older than for younger embryonic cells. Thus the growth capacity of disintegrated heart cells in culture increased with the increase of embryonic age.

The latter different growth capacity was determined by the following factors: a) a different duration of growth and b) a different growth rate.

The duration of lag (latent) period in culture was found to depend on the inoculum size, the embryonic age, and the quality of the medium.

9. To determine whether the different growth capacities displayed by heart cells of different embryonic ages are an inherent property of myoblasts, or are an artifact caused by selection of different cell types having different

proliferation rates, previously ascribed culture systems were utilized, and the differentiation potentialities of the heart cells involved were evaluated.

10. Cell cultures of all developmental ages studied were, under proper conditions, able to show a high percentage of differentiated cells. Through the action of trypsin, or simply because of the dissociation process, most of the cells that in intact heart were differentiated lost their differentiation. Depending on the quality of the medium, these cells in culture might develop in one of the following ways: a) proliferate but not differentiate, b) differentiate but not proliferate, and c) proliferate and parallelly differentiate. It was further observed that cell proliferation antagonizes cell re-differentiation, and the reappearance of differentiation during the culture period primarily depended on the rate of proliferation. A vigorous rate of proliferation completely inhibited re-exhibition of structural specificity, suggesting irreversible changes in structural proteins. However, when the cell cultures were allowed to proliferate in growth promoting conditions and then returned to the non-growth promoting conditions, the reappearance of differentiation in the latter phase was found to depend on the intensity of the proliferation in the former growth phase.

11. The EE had two separate effects on the cells in culture: a) growth promoting; b) antidifferentiative. The latter seemed to be associated with the HMW fraction of the EE. Furthermore, the effect of EE, to some extent, did not exclude enzymatic reaction, as it was found to be concentration dependent and heat labile. This suggests that the HMW fraction might contain proteolytic enzymes that decompose structural proteins, consequently resulting in de-differentiation, and inhibition of redifferentiation.

12. The ability of spontaneous contraction was shown to be a myogenic property of single cells. However, neither the cell density nor the percentage of cross-striated cells/dish was correlated with the degree of beating in a cell population.

It could happen that, in some instances during the culture period, a population of cross-striated cells does not show any contraction activity. On the other hand, it could also be noted that cultures with an insignificant percentage of cross-striated cells exhibited varying degrees of pulsating activity. Moreover, functional activity could apparently be achieved on

levels of structural organizations other than the arrangement of contractile proteins in a perfect pattern of cross-striation.

13. The morphological appearance of the nucleus proved a good indication of myogenic cells in different phases of activity. Thus, a rather small, spherical, and densely stained nucleus with predominantly one concentrated nucleolus belonged to a cell with a well-differentiated cytoplasm. On the other hand, a relatively large and not densely stained nucleus with more than two nucleoli or scattered chromatin indicated actively proliferating cells that lacked cytoplasmic differentiation. This effect was noted also in sparse cultures and in cells of prolonged cultivation, and in older cells.

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- 1. The first part of the report deals with the general situation of the country and the progress of the work.
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- 4. The fourth part deals with the conclusions drawn from the work done during the year.
- 5. The fifth part deals with the recommendations for the future.
- 6. The sixth part deals with the summary of the work done during the year.
- 7. The seventh part deals with the conclusions drawn from the work done during the year.
- 8. The eighth part deals with the recommendations for the future.
- 9. The ninth part deals with the summary of the work done during the year.
- 10. The tenth part deals with the conclusions drawn from the work done during the year.

